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Screening Methods for Probiotic Lactobacilli Isolated from the Pig Intestine

by

Jennifer Erin Snart

A thesis submitted to the Faculty of Graduate Studies and Research in the partial fulfillment of the requirements for the degree of **Master of Science**

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Abstract

Characteristics of potentially probiotic bacteria include their ability to survive the acid and bile conditions of the gastrointestinal tract (GIT) and, subsequently, colonize the epithelial layer of the intestine. A set of rapid and simple *in vitro* tests for the determination of bacterial probiotic potential were developed. These included assays of acid tolerance, bile tolerance, sequential acid and bile tolerance and several methods for the quantification of bacterial adherence. Flow cytometry was ultimately chosen as the method of choice for the indirect determination of adherence of fluorescently labeled bacteria to HT-29 epithelial cell monolayers.

Using the methods developed, the probiotic properties of *Lactobacillus* spp. isolated from the GIT of a healthy pig were evaluated. The strains studied tolerated short-term acid treatment, bile treatment and sequential acid and bile treatment. These *Lactobacillus* strains demonstrated high adherence to the HT-29 monolayers. Competition studies with *E. coli* indicated that the lactobacilli may coaggregate with *E. coli*, potentially preventing infection *in vivo*.

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1. Introduction

Animals and humans are protected from infections and disease by several mechanisms. The skin acts as a physical barrier to the external environment and potential pathogens, while the immune system can trap and kill pathogens that have gained entry to the body. The gastrointestinal tract (GIT) is unique in that it combines a physical barrier with a mucosal immune response to repel ingested pathogens. A key mechanism in the GIT's defense against infection is the host's normal intestinal microflora. Animals born in a sterile environment (gnotobiotic or germ-free animals) lack the gut microflora of conventional animals. These gnotobiotic animals have proven to be more susceptible to disease than conventionally raised animals (Fuller, 1989). Thus, microorganisms have the potential to be used in protection against infection.

In the 1950s, antibiotics were developed and used in both human and animal therapy (Fuller, 1989; 1999). Subclinical concentrations of antibiotics were added to animal feed to promote growth and health of the animals. However, in 1969, the Swann Committee in the UK recommended that antibiotics used in growth promotion of farm animals should be restricted to those that are not used therapeutically. Recent concerns regarding antibiotic resistant 'superbugs' originating from animals fed subclinical doses of anitibiotics, as well as an anti-additive lobby concerned about residues in food products, have driven the scientific community to look for alternatives to antibiotics. Fuller (1989) defined a probiotic as 'a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance'. Since then, several new definitions

have been proposed to include viable and nonviable cells as well as bacterial end products and cell components (Huis in't Veld and Havenaar, 1991). The definition provided by Fuller, however, remains the most recognized. Though not defined until 1989, the beneficial concept of probiotics has been recognized for decades. The use of *Lactobacillus acidophilus* as a dietary adjunct has increased steadily since Metchnikoff (1908) described the desirability of the organism in the intestinal tract. In 1968, King was able to stimulate growth of pigs by supplementing their feed with *L. acidophilus* (Fuller, 1999). Nurmi and Rantala (1973) found that they could prevent *Salmonella* infection in newborn chicks by dosing them with intestinal contents of healthy adult chickens. Other beneficial probiotic effects observed in animals include growth promotion and increased product weight (Fuller, 1999). Probiotic supplements have shown promise in the lowering of serum cholesterol levels, improved bioavailability of vitamins and minerals, reduction of carcinogen levels in the GIT and improvement in individuals suffering from lactose intolerance (Fooks et al., 1999; Gilliland, 1990).

Several characteristics have been identified as essential in the choice of potentially probiotic microorganisms (Garriga et al., 1998; Fuller, 1999). Probiotic organisms must be able to survive the harsh environment of the GIT, thus necessitating resistance to both acid and bile. Upon arrival in the intestine, the probiotic must be able to colonize the GIT. The organism must not only be safe for food and clinical use, but it must also exhibit documented health benefits, including inhibition of colonization of the GIT by pathogens. Finally, the organism should have good technological properties to facilitate

probiotic production and administration, including survival of freeze drying or cryopreservation.

Upon ingestion, a probiotic organism encounters the severe conditions of the GIT, including the high acidity of the stomach and the release of bile salts into the upper small intestine. The pH of the stomach has been documented to be as low as pH 1.5 in both the human and the piglet stomach (Chou and Weimer, 1999; Kidder and Manners, 1978). Normally, the pH of the stomach ranges between pH 2.5 and 3.5 (Holzapfel et al., 1998). *In vitro* studies have shown that several species and strains of *Lactobacillus* can survive exposure to such a low pH (Garriga et al., 1998; Chou and Weimer, 1999). Charteris et al. (1998) conducted a more extensive study in which *Lactobacillus* strains were subjected to a simulated gastric juice (pH 2.0) containing pepsin and sodium chloride. *Lactobacillus fermentum* KLD was the only strain of fifteen tested that was intrinsically resistant to these conditions. However, the addition of milk proteins improved the resistance of the other fourteen strains. *In vitro* resistance of *Lactobacillus* strains to bile salts has also been examined (Gilliland and Speck, 1977; Gilliland et al, 1984; Gilliland et al., 1985; Klaenhammer and Kleeman, 1981; Noh and Gilliland, 1993; Mustapha et al., 1997; Garriga et al., 1998; Charteris et al., 1998; Chou and Weimer, 1999). The concentration of bile in the upper small intestine at any one time is difficult to determine, and varies depending on diet. Generally, a bacterial strain is said to be bile tolerant if it grows on agar containing at least 0.3% Oxgall. Several studies have proven a variety of strains of *Lactobacillus* spp. to be able to tolerate and/or grow in such high bile salt

conditions (Gilliland et al., 1984; 1985; Noh and Gilliland, 1993; Mustapha et al., 1997; Chou and Weimer, 1999).

Host specificity has proven to be important in the colonizing ability of microorganisms (Fuller, 1999). Therefore, bacterial strains that have been isolated from the host animal in which its use is desired may have better probiotic potential. Before birth, a fetus is essentially sterile, but it quickly acquires a natural gut microflora upon exposure to the environment (Fuller, 1989). The newborn's gut microflora is obtained mainly from the mother and follows a natural progression until adulthood that is affected by the environment, eating habits and overall health (Fuller, 1989). Tannock et al. (1990) demonstrated that several strains of lactobacilli were common to both sow and piglet feces. The natural gut microflora of an adult can be composed of up to 400 different strains with total numbers exceeding 10^{14} microorganisms (Fuller, 1989). *L. fermentum* and *L. acidophilus* have been shown to be the most dominant lactobacilli in adult, weanling and suckling pigs (Fuller et al., 1978; Pedersen and Tannock, 1989; Naito et al., 1995). Naito et al. (1995) found that during the first 45 days of life of piglets, total bacterial counts remained $10^{9.4}$ to $10^{10.6}$ per gram of feces. However, a shift from dominant populations of *Enterobacteriaceae* and streptococci to lactobacilli was observed after the first seven days of life.

Inhibitory activity of gut microflora against a variety of pathogens, including *Escherichia coli, Salmonella, Campylobacter, Clostridium* and rotavirus has been reported (Fuller, 1999). In light of these reports, it is interesting that between 1987 and 1990 rotavirus and

E. coli were the most common pathogenic organisms isolated from nursing pigs with diarrhea (Johnson et al., 1992). *Clostridium perfringens* and several combinations of pathogens were also isolated from pigs with diarrhea. In recent years, studies have been conducted which illustrate the inhibition of enteric pathogens by lactobacilli (Hammes and Vogel, 1995; Garriga et al., 1998; Blomberg et al., 1993; Ouwehand and Conway, 1996). Blomberg et al. (1993) inhibited adherence of *E. coli* K88 to piglet ileal mucus with three different *Lactobacillus* spp. of porcine origin. *L. fermentum* 104R produces a proteinaceous compound that inhibited *E. coli* K88 adherence to ileal mucus (Blomberg et al., 1993; Ouwehand and Conway, 1996). Garriga et al. (1998) isolated several strains of *Lactobacillus salivarius* that were effective in inhibiting *Salmonella enterica* serovar Enteritidis, *E. coli* and *Campylobacter jejuni in vitro*.

If a probiotic is to be beneficial to the host it must also be nonpathogenic. Lactic acid bacteria (LAB) are ubiquitous in the environment and many of them have a long history of use in food production with no notable adverse effects in humans (Aguirre and Collins, 1993; Salminen et al., 1998). The lactobacilli are commonly assigned GRAS (generally recognized as safe) status (Salminen et al.,1998). In the past, lactobacilli found in clinical samples were often disregarded as contaminants or secondary invaders (Aguirre and Collins, 1993). The enterococci have been identified as the group of lactic acid bacteria (LAB) most likely to cause concern for the safe use of probiotic supplements (Franz et al., 1999). Although enterococci are commonly found in the gut of healthy humans and animals, they have come under scrutiny due to their resistance to several antibiotics as well as their role in clinical infections (Aguirre and Collins, 1993; Franz et al., 1999;

Salminen et al., 1998). Vancomycin resistant enterococci (VRE), for instance, have become a major concern due to their increased role in nosocomial infections and their ability to transfer the antibiotic resistance genes to other pathogens (Salminen et al., 1998). Vancomycin is a drug of last resort for the treatment of many antibiotic resistant infections. Therefore the transfer of vancomycin resistance genes may render many infections untreatable. Transmission of plasmid–mediated antibiotic resistance genes to *Listeria monocytogenes* and *S. aureus* has been demonstrated *in vitro* (Aguirre and Collins, 1993). Lactobacilli have been reported to cause dental caries, but they have otherwise been considered nonpathogenic (Aguirre and Collins, 1993). Recently, however, there has been increasing evidence that they may cause other, more detrimental, infections (Aguirre and Collins, 1993; Harty et al., 1994; Salminen et al., 1998). *Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus* and *L. acidophilus* have been associated with bacterial endocarditis (Aguirre and Collins, 1993). Other *Lactobacillus* spp. have been associated with rheumatic vascular disease, septicemia and infective endocarditis (IE) (Harty et al., 1994). A review of the literature indicates that most lactobacilli–related cases of IE were caused by *L. casei* (61.5%), whereas only 7.7% were attributed to *L. acidophilus* and no cases were attributed to *L. fermentum* (Harty et al., 1994). One of the main pathogenic traits thought to be involved in the progression of IE is the ability of the bacteria to aggregate platelets. Harty et al. (1994) found that all of the IE isolates aggregated platelets, while only half of the 'normal' oral isolates did so. Thus, it was concluded that platelet aggregation is a trait that is widespread in pathogenic lactobacilli and indicates evidence for their pathogenic potential. Although, theoretically, a safety concern may be present, it is generally

recognized that probiotic organisms, with the exception of the enterococci, pose very little threat to a healthy population. Adams and Marteau (1995), reported that no cases of *Lactobacillus*-associated infection have been traced back to a food product and that no case of clinical infection has been traced to ingested probiotic LAB. Also, local or systemic *Lactobacillus* infection is most commonly associated with individuals suffering some severe underlying condition or immunosuppression (Aguirre and Collins, 1993).

This study was designed to characterize *Lactobacillus* spp. isolated from the GIT of a pig in terms of identification properties as well as probiotic potential. Selected *Lactobacillus* spp. were used to design and compile a set of rapid and simple *in vitro* tests to determine the probiotic potential of a variety bacterial strains. These tests included acid and bile tolerance, and *in vitro* adherence to intestinal epithelial cell monolayers.

2. Review of Bacterial Adherence

2.1 Classification of *Lactobacillus* spp. and *Escherichia coli* strains

2.1.1 Classification of *Lactobacillus* spp.

Lactobacillus spp. are gram positive, non-sporeforming rod-shaped bacteria. The lactobacilli are characterized by a %G+C content of 33-55% (Stiles and Holzapfel, 1997). They are strictly fermentative and have complex nutritional requirements. *Lactobacillus* spp. are members of the lactic acid bacteria (LAB). They produce lactic acid as the sole or one of their main products of metabolism and some species also produce acetate. Therefore, these organisms are aciduric and are able to produce a pH of four in medium or food containing fermentable carbohydrate. Creating this acidic environment allows the lactobacilli to inhibit the growth of other bacteria. Several species of *Lactobacillus* have also demonstrated the ability to produce other inhibitory substances, including hydrogen peroxide, diacetyl, reuterin and bacteriocins (Ouwehand et al., 1999; Stiles and Holzapfel, 1997).

Stiles and Holzapfel (1997) indicated that there are more than fifty *Lactobacillus* spp. These organisms have classically been divided into 3 groups based on their metabolic activity. Group I lactobacilli are obligate homofermenters that ferment the majority of hexoses exclusively to lactic acid via the Embden-Meyerhof-Parnas (EMP) pathway. These strains cannot ferment gluconate or pentoses. Included in this group are *L. acidophilus* and *L. johnsonii*. *L. acidophilus* was originally isolated from infant feces in

1900, but the species has since been subdivided into six groups based on DNA homology. Group II includes the facultative heterofermenters, which ferment hexoses by the EMP pathway, but which can also ferment gluconate. They ferment pentoses using an inducible phosphoketolase enzyme, producing lactate and acetate. *L. casei* and *L. plantarum* are important food associated members of this group. *L. casei* has also been associated with the human mouth and dental plaque. *L. plantarum* is used as a starter culture in many foods, including fermented sausages. Group III are obligate heterofermenters that ferment hexoses via the phosphogluconate pathway yielding lactate, acetate or ethanol and carbon dioxide. *L. sanfrancisco* is important for flavor production in sourdough bread. *L. fermentum* and *L. reuteri* are also members of this group. Although these two species are not closely related genetically, they are often difficult to distinguish by physiological tests. It is clear that members of all three groups are important in the production of fermented foods, but Group III, in particular, are also associated with food spoilage. *L. bifermentans* causes cracking in Gouda and Edam cheeses, *L. viridescens* causes greening of cured meats and *L. brevis* spoils wine and beer (Stiles and Holzapfel, 1997).

Lactobacillus spp. have been isolated from a variety of sources including the mucosal membranes of humans and animals, plant material and fermented foods (Stiles and Holzapfel, 1997; Fuller, 1999). For instance, Holzapfel et al. (1998) indicated that the lactobacilli are one of the important bacterial populations in the stomach, duodenum, jejunum, ileum and colon of humans. Their predominance in the normal GIT microflora and their ability to inhibit the growth of other organisms indicates their favorable

inclusion in probiotic supplements. *L. acidophilus* is used in the production of acidophilus milk and *L. johnsonii* is used in yogurt with claims of health benefits (Stiles and Holzapfel, 1997).

2.1.2 Classification of *E. coli* strains

E. coli are gram negative, non-sporeforming rod-shaped bacteria. *E. coli* is an interesting bacterium because it comprises a number of strains that can have a variety of effects on the host animal. Several strains are normal inhabitants of the gut and, as such, they have been used as indicators of fecal contamination. Other strains cause a variety of diseases including diarrhea, dysentery, hemolytic uremic syndrome, septicemia and a variety of organ infections. Fitzgerald et al. (1988) studied diarrhea in young pigs, comparing the incidence of different causative pathogens over a 3-year period. While rotavirus was the most common cause, *E. coli* was a close second. A subsequent 3-year study demonstrated that *E. coli* had become the most common cause of diarrhea in young pigs (Johnson et al., 1992). Therefore, it is important to identify the virulence factors of *E. coli* strains and define methods to reduce the incidence of *E. coli* diarrhea in pigs.

Strains of *E. coli* are classified according to both serotypes and virotypes. Serotyping refers to the classification of bacteria on the basis of bacterial surface antigens. The two *E. coli* surface antigens used for classification are the somatic antigen (O) of lipopolysaccharide (LPS) of the cell membrane, and the Hauch (H) antigen of the flagellum. The O antigen identifies the serogroup, while the H antigen identifies the serotype. This method of classification has had some success in separating the

pathogenic strains from the nonpathogenic strains because some correlation exists between serogroup and virulence. A capsular (K) antigen is also sometimes used for further classification in cases where the organism is encapsulated. Virotyping is a more recent and more complex system that classifies bacteria based on mechanism and effects of attachment to host cells and the types of toxin produced. Five virotypes are currently recognized: enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EaggEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC). A sixth virotype, diffusely adherent *E. coli* (DAEC) has also been identified (Bell and Kyriakides, 1998). It has been implicated in childhood diarrhea, and fimbrial and nonfimbrial adhesins have been identified.

ETEC strains adhere to the small intestinal mucosa and produce toxins, resulting in a cholera-like disease, characterized by vomiting, diarrhea and fever. Two types of enterotoxins, heat-labile (LT) and heat-stable (ST), are produced by these organisms. LT-1 is similar to cholera toxin, while ST toxins are a family of small diarrheal toxins. ETEC-like strains have been shown to be diarrheagenic in both piglets and calves, as well as humans. EaggEC are found most often in children, causing a persistent form of diarrhea. These cells bind to the intestinal mucosa in a localized (or aggregated) pattern and produce an ST-like toxin and a hemolysin-like toxin. EPEC strains also adhere to the intestinal mucosa, but in doing so, they produce a phenomenon called attaching and effacing, in which the epithelial cells with adherent bacteria exhibit elongated microvilli and adjacent cells without adherent bacteria do not exhibit microvilli. These

ultrastructural changes result from actin accumulation and are followed by bacterial invasion and mucosal inflammation.

In contrast to both ETEC and EAggEC, the diarrhea associated with EPEC infection is a result of colonization of the mucosal surface as opposed to toxin production. EPEC strains adhere to and efface the intestinal cells of gnotobiotic piglets. EHEC strains produce a severe dysentery-like disease resembling that produced by *Shigella* in the infected host. These strains adhere to the intestinal mucosa and produce a *Shigella*-like toxin (SLT). Although several serogroups and serotypes exhibit EHEC characteristics, the predominant serotype is O157:H7. These organisms produce severe diarrhea, followed by bacteremia and multiple organ infection. The most serious sequela of EHEC strains is hemolytic-uremic syndrome (HUS) which can result in kidney failure and death. EIEC strains cause a disease indistinguishable from EHEC *Shigella*-like disease except it does not lead to HUS in the infected host. EIEC strains do not produce SLT but they invade mucosal cells and spread to adjacent cells.

2.2 Adherence of Bacterial Cells to Surfaces

Several mechanisms for bacterial adherence to surfaces have been proposed. These include nonspecific ionic and hydrophobic binding, as well as specific ligand-receptor interactions. Busscher and Weerkamp (1987) defined specific and nonspecific interactions as follows: specific interactions are microscopic interactions "between stereochemically complementary surface components occurring over the extremely short distances allowing specific ionic, hydrogen and possibly chemical bonds". Nonspecific

interactions, on the other hand, are "due to overall macroscopic surface properties" and occur over longer distances. The authors indicate that they are as yet unsure of exactly what constitutes "extremely short" and "longer" distances. In most cases, it is thought that bacterial cells employ both specific and nonspecific mechanisms to adhere closely to a particular substrate. Gusils et al. (1999b) suggested that bacterial adherence is actually a two-step event. A reversible adherence mediated by long range forces (first step), which may be followed by interactions allowing closer, more direct contact between the bacterial and tissue surfaces (second step).

In general, living cells are negatively charged due to the composition of their membranes (Bisno, 1995). On this basis, cells would naturally repel each other. Therefore, these intrinsic forces must be overcome for the bacterial cell to adhere to a tissue cell. Similarly, hydrophobic and hydrophilic surfaces will repel each other. Hydrophobic charges appear to be randomly dispersed on the cell surface (Bisno, 1995). However, if two hydrophobic regions come into close proximity they will repel water and attract each other. In this way, a bacterial cell could be brought into close contact with a tissue cell.

There are several examples of specific ligand-receptor interactions between bacteria and other biological material, including protein and carbohydrate ligands. Microbial surface components that bind to a host cell surface receptor are generally known as adhesins. Lectins, for instance, are "carbohydrate binding proteins or glycoproteins of nonimmune origin, which agglutinate cells with receptors, such as yeast and red blood cells" (Gusils, 1999a). Thus, lectins could constitute a type of adhesin that allows a bacterial cell

expressing them to bind to surfaces with exposed surface carbohydrates or glycoproteins acting as receptors. It has been proposed that these not only contribute to the adherence of the producer organisms, but they also inhibit the adherence of pathogens by competition for intestinal receptors, or steric hindrance (Gusils, 1999a, b). Electron microscopy has shown that adherence of Group A streptococci to oral epithelial cells is protein-mediated (Bisno, 1995). M protein has been identified as a surface component that protrudes into the extracellular environment in the form of multiple fimbriae. It was elucidated that M protein is an important adhesin of Group A streptococci. Further study indicated that it is a combination of lipoteichoic acid (LTA) and M protein that mediates adherence of Group A streptococci.

Fibronectin, a glycoprotein on the surface of the host tissue, has also proven to be a good receptor for the adhesion of streptococci and *Staphylococcus* spp. (Bisno, 1995). Flow cytometry techniques have been used to assess the adherence of *Helicobacter pylori* to gastric and intestinal cells (Clyne and Drumm, 1993; Logan et al., 1998). Clyne and Drumm (1993) demonstrated the specificity of *H. pylori* for gastric cells by comparing adherence to gastric cells with adherence to duodenal and colonic cells. While 70% of the gastric cells bound bacteria, only 30% and 32% of the duodenal and colonic cells, respectively, had adherent bacteria. Logan et al. (1998) also demonstrated specific adherence of *H. pylori* to gastric cells. Adherence to gastric cells was saturated after a 90-minute incubation period, suggesting a specific ligand-receptor adherence mechanism.

2.3 **Adherence Mechanisms Exhibited by *Lactobacillus***

Temporary colonization of the human gastrointestinal tract by *Lactobacillus rhamnosus* GG has been observed *in vivo* (Alander et al., 1999). Lactobacilli are also known to colonize naturally the intestinal tract of both animals and humans (Kleeman and Klaenhammer, 1982; Mäyrä-Mäkinen et al., 1983; Sherman and Savage, 1986; Tannock, 1987; Wadstrom et al., 1987; Tannock et al., 1990). *L. acidophilus*, for example, has been isolated from the intestinal tracts of humans and animals (Bisno, 1995). *L. fermentum* strains have been isolated from milk products, fermenting plant material, and the human mouth and feces (Bisno, 1995) as well as the intestinal tracts and feces of animals (Henriksson et al., 1991; Gusils, 1999b). However, relatively little research to elucidate the adherence mechanisms of lactobacilli has been reported to date. Current evidence suggests that a complex combination of factors leads to adherence and colonization by *Lactobacillus* spp. Adherence of lactobacilli exhibits a high level of host specificity (Kleeman and Klaenhammer, 1982; Sherman and Savage, 1986). However, recent studies have shown that organisms isolated from one host are able to adhere to the tissue of another. For instance, *L. acidophilus* ADH, isolated from a human, was originally shown to adhere to a human cell line, human fetal intestinal (HFI) cells (Kleeman and Klaenhammer, 1982). This same strain was later shown to bind to freshly scraped human and pig intestinal cells (Conway et al., 1987). Also, *L. reuteri* D287 isolated from the feces of a calf was shown to colonize the squamous gastric epithelium of a mouse (Sherman and Savage, 1986). *L. fermentum* strains isolated from calves adhere to both calf and porcine epithelial cells, although the adherence to the porcine

cells was weaker (Mäyrä-Mäkinen et al., 1983). Although some lactobacilli adhere to the intestinal cells of several host animals, isolates from plants, cultured milk, cheese (Mäyrä-Mäkinen et al., 1983) and other dairy isolates (Greene and Klaenhammer, 1994), generally do not. It has been suggested that adherence to cells of different origin than those of the original source may be due to nonspecific adherence, while host specific adherence is mediated by specific mechanisms (Conway et al., 1987). This would explain the decreased strength of adherence of *L. fermentum* strains to cells other than those of the original host (Mäyrä-Mäkinen et al., 1983).

Cation concentrations are thought to mediate some nonspecific binding. Calcium, for instance, is of great importance in adherence of lactobacilli (Klaenhammer, 1995). Calcium-dependence has become a characteristic of nonspecific adherence. In fact, researchers demonstrated adherence of nonadhering strains simply by the addition of calcium to the assay. For instance, of 32 lactobacilli, only four adhered to a human cell line in the absence of calcium, and they were all human isolates (Kleeman and Klaenhammer, 1982). However, all 32 strains adhered in the presence of calcium. Conway et al. (1987), Chauvière et al. (1992a) and Conway and Kjelleberg (1989) showed similar results. The addition of milk to the assay was shown to increase bacterial adherence, consistent with the enhancement of binding in the presence of calcium. Conway and Kjelleberg (1989) demonstrated that the addition of calcium could promote the adherence of *L. fermentum* 737, of rodent origin, to mouse squamous epithelial cells, but the addition of a calcium chelator, ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetaacetic acid (EGTA), did not decrease binding. These data indicate that calcium-

dependent, nonspecific adherence is different from specific adherence. It has been proposed that calcium ions may provide a bridge between the negatively charged surfaces of bacterial and epithelial cells (Kleeman and Klaenhammer, 1982).

Hydrogen ion concentration is also important in adherence (Greene and Klaenhammer, 1994; Klaenhammer, 1995). For instance, the adherence of human isolates, including *L. acidophilus* BG2 FO4, to Caco-2 cells was increased at low pH (Greene and Klaenhammer, 1994). It has been recommended that adherence studies be conducted in neutral conditions (pH 7) because the conditions of the intestine include a neutral pH (Greene and Klaenhammer, 1994). However, some researchers suggest that, *in vitro*, the metabolic activity of bacterial and tissue cells in a small experimental volume may decrease the pH of the assay (Granato et al., 1999), which could artificially increase bacterial adherence.

Surface properties of the lactobacilli that may be involved in other mechanisms of nonspecific adherence include hydrophobicity. Studies characterizing the surface properties of adherent lactobacilli indicate that high surface hydrophobicity correlates with good adherence (Wadstrom et al., 1987; Gusils et al., 1999b). Gusils et al. (1999b) found cell surface hydrophobicity to be high for strains of *L. fermentum* and *L. fermentum* subsp. *cellobiosus* isolated from chicken intestine, indicating an improved ability for these organisms to adhere to epithelial cells. Wadstrom et al. (1987) showed that porcine isolates with high surface hydrophobicity adhered better than hydrophilic strains;

however, the hydrophilic strains adhered weakly, indicating the presence of another adherence mechanism.

Specific ligand-receptor type interactions in the adherence of lactobacilli to intestinal enterocytes have only recently been identified. Lipoteichoic acids (LTAs) and surface proteins have both been implicated in specific interactions mediating the adherence of lactobacilli to intestinal epithelial cells. LTAs are amphiphilic molecules with a hydrophobic glycolipid end embedded in the cell membrane and long polar chains of glycerol phosphate that may come close enough to the surface of the bacterial cell to act as bacterial antigens (Granato et al., 1999). Sherman and Savage (1986) examined 22 strains of lactobacilli and found ten strains that contained LTA. Of these, eight strains, including strains of *L. fermentum* and *L. acidophilus*, were previously shown to adhere to mouse keratinized squamous epithelial cells. Thus, there appears to be a positive correlation between the presence of LTA on the cell surface and adherence to keratinized squamous epithelial cells. The results of Granato et al. (1999) expand this correlation to the adherence of lactobacilli to Caco-2 cells. They found that *L. johnsonii* La1 adhered to Caco-2 cells while *L. acidophilus* La10 did not. The notable difference between the two strains was the presence of LTA on the surface of strain La1. Further investigation showed that the purified LTA from these cells could inhibit the adherence of La1 cells to Caco-2 cells, indicating that this is a specific interaction.

Proteinaceous adherence components in the lactobacilli have been identified (Henriksson et al., 1991; Greene and Klaenhammer, 1994; Sarem et al., 1996; Gusils et al., 1999a;

Conway and Kjelleberg, 1989). These compounds include cell surface associated proteins and extracellular proteins. Lectins, as discussed in Section 2.2, are cell surface-associated proteins. Lectin-like proteins have been isolated from the surfaces of several *Lactobacillus* species including *L. acidophilus, L. fermentum, L. fermentum* subsp. *cellobiosus* and *L. animalis* (Takashi et al., 1996; Gusils et al., 1999a). These strains demonstrated good adherence to intestinal cells of the original host species, thus indicating that lectin-mediated adherence is host specific. Other cell surface associated proteins important in binding bacteria to intestinal epithelial cells have been identified (Henriksson et al., 1991). Adherence of *L. fermentum* 104 was reduced by protease treatment. Analysis showed that the proteins in question were tightly bound to the bacterial cell wall (Henriksson et al., 1991).

Extracellular proteins have been implicated in the adherence of strains of *L. fermentum, L. delbrueckii* subsp. *lactis* and *L. acidophilus* (Conway and Kjelleberg, 1989; Chauvière et al., 1992a; Greene and Klaenhammer, 1994; Sarem et al., 1996). Adherence of these strains was reduced when the bacterial cells were washed, or their spent culture supernatant was replaced with fresh medium. If washed cells were supplemented with spent culture supernatant (SCS), their adherence capacity was restored. Conway and Kjelleberg (1989) noted that the SCS of *L. fermentum* 737 did not improve the adherence of *Lactobacillus* strains that were not isolated from mouse squamous epithelium, indicating a host specific action of these extracellular proteins.

Current studies indicate that adherence of lactobacilli is complex and strains often use both carbohydrates and proteins to bind to intestinal epithelial cells (Chauvière et al., 1992a; Greene and Klaenhammer, 1994). *L. acidophilus* strain LB, for instance, was shown to have a two component mechanism for adherence to Caco-2 cells (Chauvière et al., 1992a). The first component is protease resistant and closely associated with the bacterial cell surface, while the second is protease sensitive, heat stable and extracellular. These data indicate that *L. acidophilus* strain LB has a proteinaceous extracellular factor, similar to those described above, but it also has a surface associated carbohydrate component that is involved in adherence. Greene and Klaenhammer (1994) reported a similar adherence mechanism for *L. acidophilus* BG2 FO4. They concluded that this strain adheres to Caco-2 cells via extracellular proteins and secondarily by cell surface associated proteins and carbohydrates.

Further study may indicate that organisms with one proposed mechanism of adherence actually employ several components for the colonization of intestinal cells. The advantages of several adherence mechanisms to the bacterial cells are many. The lactobacilli can exhibit strong adherence to a specific host, while also being able to bind to less desirable cells in a less desirable environment. Sarem et al. (1996) studied the adherence of *L. acidophilus* K1 and *L. delbrueckii* subsp. *lactis* to Caco-2 and Int-407 cells. While adherence patterns of the two bacteria were similar, adherence to the two cell lines was different. Caco-2 and Int-407 cells are both enterocyte-like cells of human origin; however, the bacterial strains used an extracellular protein to adhere to the

microvilli of the Caco-2 cells and a carbohydrate component to adhere to the Int-407 cells.

The results presented reinforce the theory that lactobacilli employ several different mechanisms to mediate both specific and nonspecific adherence. Important factors appear to be calcium dependence of nonspecific adherence and the production of extracellular proteins to mediate specific adherence. These, combined with other nonspecific forces and specific carbohydrate and protein components, allow the lactobacilli to adhere to a variety of cells, from a variety of sources, under a variety of conditions. It is also important to note that culture conditions can influence adherence of the lactobacilli. For example, bacterial strains grown on agar have exhibited better attachment than when grown in broth (Spencer and Chesson, 1994).

2.4 Adherence Mechanisms of *E. coli*

2.4.1 General Adherence Mechanisms

The adherence of *E. coli* strains has been studied more than that of the lactobacilli, because adherence of *E. coli* to intestinal epithelial cells is often considered a virulence factor. Although the nonspecific adherence mechanisms discussed previously also apply to *E. coli*, this discussion will focus on the specific interactions that have been investigated between *E. coli* and epithelial cells. ETEC-type strains have been implicated in diarrhea of calves and pigs, while EPEC strains have exhibited adhering and effacing

capabilities in gnotobiotic pigs (Salyers and Whitt, 1994). Thus, these virotypes are highly represented in the following discussion.

Adhesins of gram negative bacteria exhibit several different presentations. Fimbriae, a type of adhesin of gram negative bacteria, exhibit a defined structure and are extremely important in the adherence of *E. coli* (Bisno, 1995; Cassels and Wolf, 1995). Fimbriae are nonflagellar filaments and they function similarly to the lectins of the lactobacilli discussed in Section 2.3. Several fimbriae of *E. coli* have been identified (Bisno, 1995): types I to V, intestinal colonization factor antigens (CFAs), pyelonephritis-associated pili (P fimbriae), sialylgalactoside-binding fimbriae (S fimbriae) and fimbriae associated with virulent strains of *E. coli* (K12, K88, K99, etc). Also, the fimbrial adhesins of *E. coli* strains capable of hemagglutination can be characterized as either mannose-sensitive (MS) or mannose-resistant (MR). Mannose inhibits the hemagglutination activity of the MS strains, but not that of the MR strains. Mannose will also inhibit specific adherence mediated by MS adhesins, but not that of the MR adhesins.

Type I fimbriae are widely distributed among *E. coli* (Bisno, 1995; Cassels and Wolf, 1995; Salyers and Whitt, 1994). They are also called common fimbriae and appear to be involved in *E. coli* colonization of the urinary tract and oropharynx of animals (Bisno, 1995). Type 1 fimbriae are produced by most generic *E. coli* and are not limited to virulent strains (Salyers and Whitt, 1994). Their importance in disease is not clear (Bisno, 1995; Cassels and Wolf, 1995), but studies have shown that negative mutants lacking type 1 fimbriae remain virulent (Salyers and Whitt, 1994). Type 1 mediated

adhesion is characteristically mannose sensitive (Cassels and Wolf, 1995; Darfeuille-Michaud et al., 1990). Unlike the type 1 fimbriae, P fimbriae are clearly associated with virulent *E. coli* (Bisno, 1995). They agglutinate erythrocytes carrying the P blood group antigen. This antigen is also found on uroepithelial cells and P-fimbriated *E. coli* have been associated with pyelonephritis and urosepsis (Bisno, 1995).

2.4.2 Adherence of ETEC strains

ETEC-type diarrhea is common in cattle and pigs, therefore, much of the information about ETEC adherence and virulence mechanisms comes from studies in these animals. (Salyers and Whitt, 1994). Toxin production and action on host cells is of great importance in ETEC-associated diarrhea; however, it is thought that ETEC strains must be in intimate contact with the host tissue for the toxins to cause diarrhea (Salyers and Whitt, 1994). Eighteen colonization factors from ETEC strains have been described, including type 1 fimbriae, CFAs and a bundle-forming type of pilus, termed 'longus' due to its unusual length (Cassels and Wolf, 1995; Salyers and Whitt, 1994). Evidence suggests that these adhesins contribute to the virulence of the ETEC strains by enabling an intimate association with host tissue. CFAs, for instance, are found on ETEC strains that are capable of causing human diarrhea (Salyers and Whitt, 1994). Also, K88-positive ETEC strains produce diarrhea in piglets, while K88-negative mutants are avirulent (Bisno, 1995). K99, F41 and 987P adhesins have demonstrated roles in adherence and virulence of *E. coli* (Laux et al., 1984; Darfeuille-Michaud et al., 1990; Casey et al., 1992). In contrast, Casey et al. (1992) demonstrated that these adhesins may not be necessary to produce diarrhea in pigs. Two strains that were isolated from pigs

with diarrhea did not express any of the K88, K99, F41 or 987P adhesins, but were capable of eliciting hemolysis and hybridized to DNA probes for *E. coli* heat stable enterotoxins.

Further investigation of ETEC adherence mechanisms has shown that, similar to the lactobacilli, the ligand-receptor mediated adherence of ETEC to host cells is a specific process. ETEC expressing K88 and K99 adhesins, but not those expressing K12 or CFA/I, were shown to bind to intestinal mucosal components *in vitro*, while not binding to the bovine serum albumin controls (Laux et al., 1984). These data indicate the specificity of adherence. *E. coli* K88 strains isolated from porcine gastrointestinal tract also exhibit lectin-like activity (Meng et al., 1998). These organisms agglutinated erythrocytes, unless inhibited by protease or trypsin. Adherence mediated by these lectin-like surface proteins is thought to be separate from adherence mediated by the K88 adhesin, indicating the importance of a combination of adherence mechanisms.

Some research has also been conducted to find a suitable tissue culture cell line to study the adherence of ETEC strains. Darfeuille-Michaud et al. (1990) conducted a screening experiment to determine which cell lines allow adherence of ETEC strains. ETEC strains expressing CFA/I, CFA/II or CFA/III were screened for adherence to HeLa, HEp-2, HRT18, Hutu80, MDBK, MDCK, Vero and Caco-2 cells. Specific adherence to Caco-2 cells was exhibited. Adherence could be inhibited with specific antigens to Caco-2 cells and was specific for each of the three CFA adhesins. ETEC expressing CFA/I or CFA/II adhesins adhere specifically to the brush border of polarized Caco-2 cells (Chauvière et al., 1992b). Caco-2 cells can express CFA/I, CFA/II and CFA/III receptors once they

have differentiated into a polarized monolayer (Chauvière et al., 1992b). Other research indicates that Caco-2 and HT 29 cell lines allow the adherence of ETEC strains (Salyers and Whitt, 1994). Caco-2 and HT 29 cell lines both originated from human colon carcinomas and form differentiated, polarized monolayers with tight junctions and microvilli. Thus, these cell lines provide good models to study the mechanisms involved in ETEC adherence.

2.4.3 Adherence of EPEC strains

EPEC strains were the first *E. coli* to be recognized as important pathogens in diarrheal disease (Benz and Schmidt, 1992). Unlike ETEC strains, EPEC strains cause diarrhea mainly due to adherence to host intestinal epithelial cells, not toxin production. However, the diarrheagenic mechanism appears to be more complex than that of ETEC strains. Perhaps due to this complexity, the adherence of EPEC is not clearly understood (Benz and Schmidt, 1992). Three stages of interaction of EPEC with host cells have been defined (Cassels and Wolf, 1995; Salyers and Whitt, 1994). The first step is nonintimate binding mediated by bundle-forming pilus (BFp). BFp may also be responsible for bacterium-bacterium interactions, or coaggregation. The second step is characterized by bacterial attachment followed by an increase in host cell intracellular calcium levels. Phosphorylation of host cell proteins has also been reported (Salyers and Whitt, 1994), which may disrupt signal transduction in the cell. EPEC strains also decrease the transepithelial electrical resistance across polarized monolayers of both Caco-2 and MDBK cells (Canil et al., 1993). During the third step, the bacterium exhibits intimate contact with the host cell and actin filaments rearrange and aggregate in the region of

bacterial attachment forming a "pedestal-like" structure. Actin accumulation has also been noted during the adherence of segmented filamentous bacteria to mouse ileal epithelial cells (Jepson et al., 1993) EPEC strains have an outer membrane protein, intimin, which is important in the intimate attachment of bacteria to epithelial cells in culture (Bisno, 1995). Steps two and three constitute the attaching and effacing property characteristic of EPEC strains. Although most strains of EPEC adhere to intestinal epithelial cells in a localized or patchy pattern, some strains exhibit a diffuse adherence phenotype. Benz and Schmidt (1992) investigated the adhesin involved in diffuse adherence (AIDA-I) of a diarrheagenic *E. coli* strain 2787. They discovered that this adhesin is synthesized via a precursor molecule and that it is, at least partially, responsible for the diffuse adherence phenotype. Another adhesin, F1845, was described by Yamamoto et al. (1994) and is thought to mediate diffuse adherence of nonEPEC strains. Research indicates that the diffuse adherence pattern may be associated with a lack of actin accumulation at the site of bacterial adherence. (Yamamoto et al., 1994).

Scaletsky et al. (1996) investigated the virulence mechanisms of the EPEC strain 018ab isolated from a patient with persistent diarrhea. Using an *in vitro* model of HeLa cell monolayers, they demonstrated that the strain may be able to cause persistent diarrhea by attaching to and effacing intestinal tissue, and subsequently invading the bound epithelial cells. Adhesins including K88 are found in EPEC strains as well as ETEC strains (Blomberg et al., 1993; Hu et al., 1993). K88 adhesins mediate adhesion of EPEC strains to porcine enterocytes, as well as porcine intestinal mucus (Blomberg et al., 1993; Hu et al., 1993). As in ETEC pathogenesis, the adhesins mediate host specific attachment to

intestinal epithelial cells. The host animal also plays a role in the specificity of adherence. Hu et al. (1993) demonstrated that expression of small intestinal receptors by porcine enterocytes mediating adherence of EPEC K88 was variable. Therefore, a bacterial strain possessing particular fimbriae may differentially colonize host animals based on receptor expression. Genetics of the host will play a role in host susceptibility to *E. coli* colonization and diarrheal disease.

In contrast to ETEC strains, EPEC strains adhere to HEp-2 and HeLa cell lines *in vitro* (Salyers and Whitt, 1994). Unlike Caco-2 and HT 29 cell lines, these cell lines are undifferentiated. HEp-2 cells in particular, exhibit the same structural response to EPEC adherence as intestinal cells *in vivo*. That is, they undergo the same attaching and effacing phenomenon. Therefore, these cell lines provide a good model for examining the adherence and virulence of EPEC strains. EPEC strains also adhere to the brush border of differentiated Caco-2 cells (Coconnier et al., 1993). It is interesting to note that *Listeria monocytogenes* strains only adhere to the periphery of undifferentiated Caco-2 cells (Coconnier et al., 1993).

2.5 Competition Between *Lactobacillus* spp. and *Escherichia coli*

It has long been thought that the natural microflora in the gastrointestinal tract of a host may be beneficial in protecting against intestinal infection and disease (Metchnikof, 1908). Nurmi and Rantala (1973) prevented *Salmonella* infection in newborn chicks by dosing them with the intestinal contents of healthy adult chickens. Mann et al. (1980) were able to protect six day-old gnotobiotic lambs from *E. coli* associated illness by

dosing the animals with either *L. casei* LB17 or *L. acidophilus* (Mann et al., 1980). Carriage of *E. coli* O157:H7 in cattle was effectively reduced by inoculation of calves with nonpathogenic strains of *E. coli* isolated from healthy cattle (Zhao et al., 1998). The protected animals did not exhibit signs of disease for the 25 to 27 day period of the experiment. These data support the theory that nonpathogenic bacteria may protect a host from intestinal disease. Zani et al. (1998) demonstrated that CenBiot (a probiotic formula containing *Bacillus cereus*) was as effective in reducing the incidence of *E. coli* induced piglet diarrhea as a commonly used antibiotic Furazolidone.

The phenomenon of competitive exclusion remains relatively unclear, but recent studies have advanced the understanding of mechanisms that may be involved. For instance, lectin-like activities have been observed in both *E. coli* K88 and *Bifidobacerium pseudolongum* isolated from the porcine gastrointestinal tract (Meng et al., 1998). These findings indicate that pathogens and natural gut microflora employ similar mechanisms of adherence. Therefore, maintenance of a predominantly beneficial gut microflora may prevent colonization by a pathogen.

Studies have shown that living cells and cell wall preparations inhibit the adherence of *E. coli* to epithelial cells *in vitro* (Chauvière et al., 1992b; Chan et al., 1985; Coconnier et al., 1993). The inhibitory organism may prevent the adherence of pathogenic *E. coli* by inhibiting contact with the intestinal epithelial cells and associated receptors. *L. acidophilus* strain LB, for instance, has previously been shown to adhere to Caco-2 cells *in vitro* (Chauvière et al., 1992b). Heat killed LB cells also inhibit ETEC expressing

CFA/I and CFA/II adhesins from binding to the brush border of the Caco-2 cell monolayer (Chauvière et al., 1992b). *L. acidophilus* does not express these ETEC adhesins; therefore, the competitive exclusion is thought to be due to steric hindrance. A subsequent experiment by Coconnier et al. (1993), showed that live *L. acidophilus* strain LB cells and heat killed cells inhibited the adherence to and invasion of Caco-2 cells by EPEC strains in a concentration dependent manner. Steric hindrance was again implicated as the mechanism of exclusion. A *Lactobacillus* strain isolated from the urethra of a healthy woman was shown to inhibit the adherence to uroepithelial cells of uropathogens, including mannose-sensitive and mannose-resistant *E. coli* strains, as well as capsulated *E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Chan et al., 1985). The variety of organisms that are inhibited by this single strain of *Lactobacillus* indicates that steric hindrance acts in a nonspecific way to inhibit adherence of other bacteria.

L. fermentum strain 104R, the rough variant of strain 104, inhibited the adhesion of *E. coli* K88 to piglet ileal mucus (Blomberg et al., 1993). Spent culture supernatant of *L. fermentum* 104R inhibited the adhesion of *E.coli* K88ab strain G1108E and *E. coli* K88 strain 1107 to 35-day-old piglet ileal mucus. This inhibitory mechanism was mediated by interaction of the extracellular compound with mucous components, therefore, specifically inhibiting the association of fimbriae with their receptors. Specificity of this mechanism is also indicated by the fact that *L. fermentum* 737 of rodent origin was unable to inhibit the adherence of strain G1108E. The extracellular inhibitory compound was initially thought to be proteinaceous, but Ouwehand and Conway (1996) proposed

that it was a carbohydrate containing glucose, N-acetylglucosamine and galactose, and that it probably originated from the cell wall of lysed cells. Although a specific association between the inhibitory component and proteins in the porcine ileal mucus was proposed, the inhibitory mechanisms remain unclear and may be due to steric hindrance.

Reid et al. (1988) demonstrated that lactobacilli, including *L. casei* ssp. *rhamnosus* GR-1 and *L. fermentum* A-60, could aggregate with type 1 fimbriated *E. coli* ATCC 25922 and strain 2239, as well as P fimbriated *E. coli* 917. It was hypothesized from these results that coaggregation of lactobacilli to uropathogens may be an important protective mechanism against the colonization of epithelial cells with pathogens.

The results presented provide a wealth of research indicating that the lactobacilli can prevent adherence of *E. coli* strains to epithelial cells. However, it is also necessary to understand that not all of the research supports these observations. Clements et al. (1981), for instance, investigated the efficacy of Lactinex® (a commercial probiotic product) in preventing diarrhea due to ETEC infection *in vivo*. Lactinex® is a combination of dried *L. acidophilus* and *L. bulgaricus*. The probiotic product was unable to prevent or alter the course of ETEC diarrhea in adult volunteers. However, ETEC diarrhea is caused, in part, by toxin production. If intimate association between ETEC and host intestinal epithelial cells is partly inhibited, ETEC strains will still produce their potent enterotoxins. Spencer and Chesson (1994) used an *in vitro* model of porcine enterocytes to demonstrate the competitive exclusion of ETEC by strongly adherent *L. fermentum* strains. These strains did not prevent the attachment of ETEC strains to

isolated porcine enterocytes in competitive exclusion, competition or displacement studies; however, the lactobacilli were shown to coaggregate with *E. coli* K88 strains. Coaggregation was suggested as a possible defense and clearance mechanism *in vivo*. Under the assumption that inhibition of *E. coli* adherence by lactobacilli is mediated by steric hindrance, there may be a minimum number of lactobacilli adhering to the tissue that are necessary to inhibit the adherence of *E. coli*. Also, this mechanism may not function in displacing attached *E. coli*.

2.6 *In vitro* vs. *In vivo* Measurement of Bacterial Adherence to Intestinal Cells

Opinions of the use of *in vitro* assays to represent *in vivo* conditions have been conflicting. The environment *in vivo* is complicated and involves several components that are not always reproducible *in vitro*. However, *in vitro* models are invaluable in the investigation of specific interactions and effects. Several *in vitro* model systems have been identified for the study of bacterial adherence (Ofek and Doyle, 1994). They vary in their representation of natural target surfaces. Solid surfaces coated with substances with different chemical properties may be used to characterize adherence to hydrophobic surfaces, but poorly represent the *in vivo* environment as a whole. Surfaces coated with components from the environment in question have been used to assess adherence of lactobacilli to mucosal components and ileal mucus. For instance, hemagglutination assays are commonly used to screen for lectin-like components in the cell surfaces of bacterial cells (Takashi et al., 1996). Hemagglutination of erythrocytes and yeast indicate the presence of lectins in the bacterial surface. Takashi et al. (1996) identified another

method for the detection of lectins. They proposed that rat colonic mucin could be used effectively in place of either erythrocytes or human colonic mucin.

Scraped intestinal cells and pieces of tissue isolated from the GIT provide better models of the *in vivo* conditions because they are the target cells of interest. *L. fermentum* 737 adherence, for instance, was assessed using pieces of stomach squamous epithelium in a bacterial suspension (Conway and Kjelleberg, 1989). The analysis of bacterial adherence to tissues excised after *in vivo* inoculation may provide a model closest to the natural conditions because adherence is occurring *in vivo*. However, sources of intestinal tissues, especially from humans, are scarce and excision is labor intensive.

Continuous cell lines are also available to study bacterial adherence. However, host specificity exhibited *in vivo* has classically limited the use of cell lines for bacterial adherence. Recent studies indicate that there is potential for several cell lines to be used to represent mammalian cells. Selection of the cell line is dependent on the bacterial strain and which aspects of adherence are to be examined. For instance, EPEC strains are known to adhere to Hep-2 and HeLa cell lines, while ETEC strains adhere to Caco-2 and HT-29 cell lines (Salyers and Whitt, 1994). Caco-2 and HT-29 cell lines form polarized monolayers with tight junctions between cells and microvilli on their apical surfaces. In this way, they are better models for intestinal tissue than HeLa or HEp-2 cells lines. *Lactobacillus* spp. have demonstrated adherence to a human fetal cell line (Kleeman and Klaenhammer, 1982) and Caco-2 cell line (Chauvière et al., 1992a). Chauvière et al. (1992a) indicated that adherence of lactobacilli to Caco-2 cells increases as the cell line

differentiates due to the expression of specific *Lactobacillus* binding sites upon differentiation of the cell line. HEp-2 and HI cell lines express receptors for F1845 fimbriae that are responsible for diffuse adherence of certain *E. coli* strains (Kernéis et al., 1991). However, cell lines may present problems due to the heterogeneity of the cell population, surface changes due to *in vitro* culture and the lack of mucus formation normally associated with intestinal cells *in vivo* (Kleeman and Klaenhammer, 1982).

Once an *in vitro* model has been selected, a quanititation method must be chosen. A common method used in conjuction with scraped epithelial cells and epithelial cell lines is direct microscopic evaluation. Giemsa staining (Kleeman and Klaenhammer, 1982) or gram staining (Mäyrä-Mäkinen et al., 1983) followed by light microscopy are documented techniques. Other researchers have radioactively labeled experimental strains of bacteria and examined radioactive counts as an indirect evaluation of adherence (Conway et al., 1987; Greene and Klaenhammer, 1994). Spectrofluorometry has been used to quantitate the adherence of fluorescently-labeled *Pasteurella haemolytica* to bovine epithelial cells (Clarke et al., 2000). Also, flow cytometric methods have been used to quantitate the adherence of fluorescently-labeled *Helicobacter pylori* to gastric epithelial cells (Logan et al., 1998). Flow cytometry provides a direct method for studying cells by fluorescence-activated cell sorting (FACS). An extensive amount of information about the cell population being studied can be obtained using flow cytometry. When a fluorescently-labeled compound is excited by a light source, the light can be absorbed and emitted at a different wavelength (fluorescence) or it can be deflected forward or at a 90° angle from the compound without a change in wavelength

(forward and side scatter). These three parameters are all measured by flow cytometry. Fluorescence data can be used to quantify cells, or events, and sort them into groups based on fluorescence emission. Scatter is proportional to size, shape and optical homogeneity of cells. Forward scatter is related to cell size and can be used to differentiate live and dead cells. Side, or 90°, scatter is related to cell granularity and can be used to differentiate granular cells (i. e., granulocytes) from nongranular cells (i. e., platelets). Flow cytometry is a rapid, specific and sensitive method to sort and assess cell populations and may be an important tool in the investigation of bacterial adherence to epithelial cells.

Conflicting data exist for the extrapolation of *in vitro* data to potential *in vivo* effects. Conway et al. (1987) demonstrated that the adherence of lactobacilli *in vitro* reflected their observations of adherence *in vivo*. Also, several studies have demonstrated that bacterial strains isolated from particular host animals can colonize tissue or cells isolated from the same animal. This indicates that adherence to intestinal cells *in vitro* may be indicative of the ability to adhere *in vivo* across a narrow range of host species. However, it is important to note that colonization of the GIT and subsequent beneficial or detrimental effects requires several interactions, including, but not limited to, adherence of the bacterial strain to intestinal tissue. Therefore, extrapolating *in vitro* adherence data to *in vivo* probiotic or pathogenic effects may be difficult. For instance, preliminary studies using Lactinex® *in vitro* indicated that these lactobacilli could colonize the small intestines of nonfasting volunteers for up to six hours. These data indicated that the probiotic may be able to prevent adherence and subsequent diarrhea from ETEC strains.

However, the lactobacilli were unable to prevent diarrhea in adults. In this case, it is important to note that toxin production is an important virulence factor of ETEC strains.

In order to maximize the use of *in vitro* models to represent *in vivo* conditions for bacterial adherence to intestinal tissues the pH should be neutral and ion concentrations should be standardized. *In vivo* conditions are complex and may be impossible to reproduce *in vitro*. However, a combination of a range of different *in vitro* models could contribute greatly to the overall understanding of bacterial adherence mechanisms.

3. Materials and Methods

3.1 Bacterial Cultures and Culture Media

Experimental strains of *Lactobacillus* spp. are listed in Table 1. Stock cultures of strains of *Lactobacillus* and *Escherichia coli* were maintained at –70°C in Lactobacilli MRS (MRS) and Nutrient broth (Difco Laboratories, Detroit, MI, USA), respectively, containing 20% v/v glycerol as a cryoprotectant. All cultures were propagated twice before experimental use. Sterile MRS broth (Difco) and agar (1.5% w/v) (Difco) were used to propagate *Lactobacillus* spp. APT broth (Difco) and agar (1.5% w/v) (Difco) were used in the initial characterization of the *Lactobacillus* spp. Nutrient broth (Difco) and Nutrient agar (Difco) were used to propagate the strains of *E. coli*. All prepared media were stored at 4°C. To assess acid tolerance, HCl was added to MRS broth to produce acidified MRS at pH 1.5, 2.5 and 3.5. Unacidified MRS broth has a pH of 6.8. To assess bile tolerance, increasing concentrations of Oxgall (Difco) were added to MRS broth to obtain bile-containing MRS broth (bMRS).

3.2 Plasmid Isolation and Profiling

Small-scale plasmid isolation was used for preliminary characterization of the bacterial cultures, using methods modified from those of Frère (1994).

3.2.1 Plasmid Isolation

Experimental strains were inoculated (1%) into MRS broth and incubated at 37°C for 18 to 24 hours. A total volume of 6 ml of each bacterial culture was centrifuged in an

Table 1. Strains of *Lactobacillus spp.* used in the study

Strain*	Comments
23IM**	Isolated from ileum
25IM**	Isolated from ileum
22IM	Isolated from ileum
27IM	Isolated from ileum
27JM**	Isolated from jejunum
23JK	Isolated from jejunum
24JC	Isolated from jejunum
25CC	Isolated from cecum
24CK	Isolated from cecum
25CK	Isolated from cecum
22CM	Isolated from cecum
$22CM_{B1}$	Isolated from cecum
$22CM_{B2}$	Isolated from cecum
24PK	Isolated from pars esophagea
26PC	Isolated from pars esophagea
21PM**	Isolated from pars esophagea
26LM**	Isolated from large intestine
21LC	Isolated from large intestine
22LC	Isolated from large intestine
L. acidophilus BG2 FO4	Exhibits good adherence to intestinal cells (Kleeman and Klaenhammer, 1982)
L. plantarum 4008	Preliminary studies showed little/no adherence to HT29 monolayers

*Experimental strains were isolated from a healthy pig

**Strains were selected for one or more of the following: acid and bile tolerance, sequential acid and bile tolerance, adherence to HT29 monolayers and inhibition of *E. coli* adherence

Eppendorf centrifuge (model 5415C, Brinkman Instruments, Inc., Westbury, NY, USA) at 6500 rpm for 4 min in 1.5-ml propylene Eppendorf tubes (Fisher Scientific, Nepean, ON). The pellet was resuspended in 300 µl of solution I (10 mM EDTA, 50mM Tris pH 7.5) at 4°C and 200 to 300 mg of 0.106-mm glass beads (Sigma Chemical Co., St. Louis, MO, USA) were added to the Eppendorf tube. The contents of the tube were mixed with a vortex (Fisher) for 30 seconds at maximum speed, before the addition of 300 µl of freshly prepared solution II (0.2M NaOH, 1% w/v SDS), and incubated at room temperature for 5 minutes. A 300-µl volume of solution III (2.55M potassium acetate, pH 4.8) was added to each tube and centrifuged at 10,000 x *g* for 5 minutes. Supernatant was transferred to two new tubes (approximately 400 µl each). Phenol (200 µl) was added to the tube, mixed and pulse centrifuged at 14,000 rpm (Brinkman) for 2 seconds. Chloroform (200 µl) was added to the tube, mixed and centrifuged at 14,000 rpm (Brinkman) for 10 minutes. Supernatant was transferred to a sterile tube and 200 µl chloroform:isoamyl (24:1) was added prior to mixing and centrifuging at 14,000 rpm (Brinkman) for 5 minutes. Finally, the supernatant was transferred to a new tube and mixed with 33 µl of 3 M sodium acetate and 700 µl of 95% v/v ethanol and stored overnight at –20°C. Once the DNA was precipitated, the tubes were centrifuged at 14,000 rpm (Brinkman) for 15 minutes. The DNA pellet was washed carefully by inversion with 700 µl of 70% v/v ethanol and centrifuged at 14,000 rpm (Brinkman) for 10 minutes. The ethanol was removed and the residual ethanol was evaporated under vacuum for 5 minutes. Duplicate tubes were combined and the pellets were resuspended in 10 µl of Milli-Q water for analysis by gel electrophoresis or storage at –20°C.

3.2.2 Gel Electrophoresis

Agarose gels were prepared using 0.8% w/v agarose (Life Technologies, Inc. Gathersburg, MD, USA) and 4 µl of ethidium bromide (10mg/ml) (BioRad Laboratories, Hercules, CA, USA) in 100 ml TAE buffer (0.04M Tris-acetate, 0.001M EDTA). The 10-µl samples were mixed with 2 µl of loading buffer before being pipetted into the wells of the gel, and submerged in TAE buffer in a gel electrophoresis unit (Tyler Research Instruments). A control DNA ladder was produced using Lambda DNA, digested with *Cla*I, providing known sizes of DNA fragments. Also, *Carnobacterium piscicola* UAL 8 was used as a control for the plasmid extraction. The loaded gel was subjected to a constant voltage of 24 volts supplied by an Electrophoresis Power Supply (BioRad), for 16 to 24 hours. The DNA was then visualized using either a UV light box (Fotodyne Inc, New Berlin, WI, USA) and MP-4 Land Camera and film (Polaroid), or a Gel Doc 1000 and accompanying Molecular Analyst software (Bio-Rad).

3.3 Physiological Tests on Experimental Cultures

3.3.1 Growth on Lactobacilli MRS Agar

Bacterial strains were plated onto MRS agar and incubated anaerobically at 37°C for 18 to 24 hours. Colony formation indicated the possible presence of lactobacilli.

3.3.2 Gram Staining

Experimental strains were inoculated onto MRS agar plates and incubated anaerobically at 37°C for 18 to 24 hours. Colonies were picked from the agar, mixed with single drops

of sterile distilled water and heat fixed onto microscope slides. The Gram reactions of the experimental strains were subsequently assessed using standardized solutions provided in BBL Gram staining kits (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). The slides were flooded with crystal violet and incubated at room temperature for 20 seconds. The samples were washed with Milli-Q water and stained with Gram's iodine for 60 seconds. Samples were washed with decolorizing solution for 1 to 2 seconds and rinsed with Milli-Q water before a final 20-second staining with safranin solution. The stained, rinsed and dried slides were then observed with a light microscope under oil immersion at 100X magnification to identify gram-positive bacilli.

3.3.3 Catalase Reaction

Twenty-four-hour colonies grown on MRS agar at 37°C were used to assess the catalase reaction. A sterile loop was used to smear some of the colony in a drop of 20% v/v hydrogen peroxide. A positive reaction was indicated by the formation of bubbles.

3.3.4 API Identification Strips

Fermentation profiles of possible *Lactobacillus* spp. were determined by API 50 CHL identification strips (bioMerieux Vitek Inc., France) according to the manufacturer's instructions. For the API strip test, overnight cultures of selected presumptive *Lactobacilli* spp. were centrifuged at 7,710 x *g* for 10 min at 4°C [Sorvall® RC-5B Refrigerated Superspeed Centrifuge (Mandell Scientific Co. Ltd.)]. The pellets were washed and resuspended in 2 ml of sterile Milli-Q water. This cell suspension was used

to adjust the OD_{600} of 5 ml of sterile Milli-Q water to 2 McFarland units (~OD_{600} = 0.35) using a Spectronic 21 spectrophotometer (Bausch & Lomb, USA). Twice this amount of cell suspension was added to 10 ml of CHL medium (bioMerieux), which was used for inoculating the API strips. The test strips were incubated at 37°C and color changes were monitored after 3, 6, 24 and 48 hours of incubation. The resulting carbohydrate fermentation profiles were submitted to API (bioMerieux Canada, Inc., St-Laurent, Quebec.) and the *Lactobacillus* spp. were identified by matching fermentation profiles of the experimental strains to existing profiles in an API database.

3.3.5 Bacteriocin Production

Selected *Lactobacillus* spp. were assayed for the production of bacteriocins using direct and deferred inhibition, and spot-on-lawn assays.

a) Direct and Deferred Inhibition:

Overnight cultures were spotted onto MRS agar using a replica plater. Once the spots dried, the plates were either immediately overlayered (direct inhibition) with 10 ml of soft MRS agar (0.8% agar) containing 1% of the indicator organism or incubated at 37°C until visible colonies formed prior to overlayering (deferred inhibition) with the indicator organism. All plates were incubated at 37°C for 24 hours prior to assessment of inhibition of bacterial growth.

b) Spot-on-lawn:

A 10-ml volume of soft MRS agar (0.8% agar) was inoculated with an overnight culture of the indicator organism and poured over a MRS agar plate. The supernatant of a 24-hour culture of the test organism was heated in boiling water for 6 minutes to obtain cell-

free supernatant (CFS). This ensures that any growth observed on the plate after spotting is the indicator, rather than the producer strain. Once the soft agar has solidified, 5 µl of the CFS was spotted onto the indicator lawn and allowed to dry in a laminar flow hood. All plates were incubated at 37°C for 24 hours prior to assessment of inhibition of bacterial growth.

3.4 Acid Tolerance

3.4.1 Plate Count Method

Initially, a plate count method was used to assess the acid tolerance of the selected *Lactobacillus* spp. Experimental strains were incubated in MRS broth at 37°C for 18 to 24 hours. Cultures were inoculated (1%) into 10-ml volumes of MRS broth (pH 6.8) and acidified MRS (pH 1.5, 2.5 and 3.5). Aliquots were sampled at time 0, 1.5, 3 and 24 hours, diluted and plated onto unacidified MRS agar (pH 6.8). The plates were incubated at 37°C for 24 hours and colonies were counted.

3.4.2 Absorbance Method

Using a method modified from that of Chou and Weimer (1999), the acid tolerance of the selected *Lactobacillus* spp. was evaluated. Experimental strains were incubated in MRS broth at 37°C for 18 to 24 hours. Cultures were inoculated (1%) into control MRS (pH 6.8) and acidified MRS (pH 1.5, 2.5 and 3.5) in 96-well plates. Bacterial survival and growth were quantified by observing changes in OD_{650} using a THERMOmax microplate

reader and accompanying SOFTmax™ software (Molecular Devices, Menlo Park, CA, USA). Absorbance was observed for up to 48 hours.

3.5 Bile Tolerance

3.5.1 Preliminary Bile Tolerance

Experimental strains were incubated in bMRS (1% inoculum) with a range of Oxgall concentrations (0, 0.15, 0.3, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0%). Bile tolerance was assessed by visual monitoring of growth at 37°C over a period of 72 hours.

3.5.2 Bile Tolerance

Using a method modified from Chou and Weimer (1999) and Vaheri (1996) the bile tolerance of selected *Lactobacillus* spp. was assessed. Experimental strains were incubated in MRS broth at 37°C for 18 to 24 hours. The cultures were inoculated (1%) into bMRS containing a range of Oxgall concentrations (0, 0.3 and 1.5% w/v) in 96-well plates. Optical density (OD) at 650 nm was observed, using a microplate reader (Molecular Devices), for a period of up to 48 hours to monitor bacterial survival and growth.

3.6 Sequential Acid and Bile Tolerance

A method modified from Chou and Weimer (1999) was used to mimic the passage through the gastrointestinal tract (GIT). Selected *Lactobacillus* spp. were incubated in MRS broth at 37°C for 18 to 24 hours. MRS broth (pH 6.8 and pH 3.5) was inoculated

(1%) and incubated at 37°C for 1.5 hours in a 96-well plate. OD at 650 nm was monitored using a microplate reader (Molecular Devices). The plate was removed from the microplate reader after 1.5 hours and the acidic broth cultures were neutralized to a pH of 6.8 using a predetermined volume of 5 N NaOH. The final pH was confirmed using pH paper. To one set of wells at each initial pH, the equivalent of a final concentration of 0.2% w/v Oxgall was added. The 96-well plate was returned to the microplate reader and the OD_{650} at 37°C was determined for at least another 24 hours.

3.7 Bacterial Adherence to a Human Intestinal Epithelial Cell Line

Adherence of selected *Lactobacillus* spp. to monolayer cell culture was assessed using fluorescently labeled bacteria visualized microscopically and quantified using a flow cytometer.

3.7.1 Human Cell Line

HT-29 cells (ATCC HTB-38) are colon carcinoma cells of human origin. They were used to study the adherence of selected bacterial cultures. The cells were used at passage levels of 2 to 17. The cells were cultured in a monolayer with growth medium of McCoy's 5A medium (Gibco BRL Products, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS) (Gibco).

3.7.2 Preparation of Tissue Culture Medium

McCoy's 5A (Gibco) was prepared according to manufacturer's instructions. Sodium bicarbonate (2.2 g/L) was added and the pH was adjusted to pH 7.3. The medium was

filter-sterilized using a Sterivex™-GP 0.22 μm filter unit with a filling bell (Millipore Corp, Bedford, MA, USA) and a peristaltic pump (Sorvall®, Newtown, CT, USA) and supplemented with 10% v/v FBS. Prepared medium was stored at 4°C and used within 6 weeks.

3.7.3 Growth and Maintenance of Cell Culture

HT-29 cells were routinely cultured in 75 cm^2 disposable sterile tissue culture flasks (Sarstedt, Inc, Newton, NC, USA). The cells grew in tissue culture medium at 37°C in 10% v/v CO_2 to reach confluent monolayers before use or transfer. Confluence was assessed using a PhotoZoom™ Inverted Microscope (Bausch & Lomb, USA). For use in adherence experiments, the monolayer of cells was rinsed with 5 ml of sterile phosphate buffered saline (PBS) at pH 7.4 (Gibco) followed by 3 ml of trypsin:EDTA (0.25% w/v trypsin, 1 mM EDTA-4 Na) (Gibco). Cells were detached by incubation at 37°C with a 5-ml volume of trypsin:EDTA for 5 to 10 minutes. A 10-ml volume of cell culture medium was mixed with the detached cells. An 8-ml volume of this suspension was transferred to 42 ml of fresh cell culture medium and was distributed, 2 ml/well, into a 24-well plate (Costar Corning Inc, Corning, NY, USA). The remaining cell suspension was inoculated into 35 ml of cell culture medium to continue the cell culture. The 24-well plate and the flask were subsequently incubated at 37°C under 10% v/v CO_2 until the cell monolayers became confluent.

3.7.4 Fluorescent Staining of Bacterial Strains

Fluorescent staining was performed using methods modified from Clarke and Morton (2000). Preliminary experiments were done to determine the final concentration of fluorescein isothiocyanate (FITC; Molecular Probes Inc., Eugene, OR, USA) to be used in adherence experiments. Selected *Lactobacillus* spp. were incubated at 37°C in MRS broth for 16 to 24 hours. A 6-ml volume of each culture was centrifuged in an Eppendorf centrifuge (model 5415C, Brinkman Instruments, Inc.) at 6500 rpm for 4 min in 1.5-ml propylene Eppendorf tubes (Fisher). The bacterial pellets were washed once with sterile PBS and resuspended in 1-ml PBS containing 0.05 mg/ml FITC. The suspensions were incubated at 4°C for 1 h before washing three times with 1 ml sterile PBS to remove residual FITC stain. Clarke and Morton (2000) determined that three washes were sufficient to remove residual FITC from the bacterial pellet. Preliminary studies were conducted to ensure that three washes would be adequate in this study, in order to prevent the staining of the HT29 monolayers.

3.7.5 Adherence of Selected *Lactobacillus* spp. and *Escherichia coli* to HT29 Monolayers

Confluent HT29 monolayers were grown in 24-well plates, as described above. All 24 wells were washed once with 1 ml of sterile PBS prior to the addition of 1 ml of FITC-labeled bacterial suspension to each of the wells. The plates were incubated for 30 minutes at 37°C to allow for adherence to the HT29 monolayers. Each well was subsequently washed three times with 1 ml of sterile PBS to remove nonadherent bacteria.

3.7.6 Fluorescence Microscopy

Fluorescence microscopy was initially used as a preliminary screening technique to determine the utility of the fluorescent staining technique for lactobacilli and *E. coli*. Also, fluorescence microscopy of several FITC-labeled *Lactobacillus* strains enabled the identification of positive and negative adherence controls. The strains screened to identify a useful negative control are listed in Table 2. HT-29 cell monolayers were grown on circular glass coverslips in the 24-well plate using methods modified from those of Wu et al.. The cell monolayers and adherent bacteria were fixed to the coverslips by incubation with 1 ml/well of methanol at room temperature for 15 minutes. The methanol was removed and replaced with 0.5 ml of sterile PBS. The coverslips were subsequently removed from the 24-well plates. FITC has an excitation wavelength of 494 nm and an emission wavelength of 520 nm. Visual assessment using a Zeiss Axioskop 20 incident-light fluorescence microscope (Carl Zeiss, Microscope Division, D-73446 Oberkochen, Germany) was used for a preliminary, visual assessment of the adherence ability of selected lactobacilli.

3.7.7 Method Development for Direct and Indirect Assessment of Adherence

Several methods for the indirect assessment of bacterial adherence to HT-29 monolayers were developed and tested before finding optimal evaluation conditions. As indicated in section 3.7.6, microscopic evaluation was used initially to assess bacterial adherence. For quantitative assessment, it was proposed to count directly the number of bacterial cells adhering per HT-29 cell. Documented methods (Kleeman and Klaenhammer, 1982; Mäyrä-Mäkinen et al., 1983) were modified for use with fluorescently-labeled bacteria

Table 2: Screening of *Lactobacillus* spp. for High and Low Adherence Control Strains

***Lactobacillus* Strain**	**Adherence and Background Fluorescence**
L. acidophilus 33193	High adherence and high background*
L. acidophilus 33199	Some adherence and low background
L. acidophilus 43121	Some adherence and low background
L. casei 53103	Some adherence and high background
L. confusus 10881	Poor adherence and low background
L. curvatus 25601	Some adherence and high background
L. plantarum 4008	Very poor adherence and some background
L. plantarum 8014	Very poor adherence and high background
L. plantarum 14917	Some adherence and high background
L. acidophilus BG2 F04	Very good adherence and low background
HT-29 cells alone	No adherence and low background

*background represents fluorescence from HT-29 cells and residual FITC stain

and evaluation by fluorescence microscopy. Secondly, a Luminescence Spectrometer (model LS 50B, Perkin Elmer Ltd., UK) and corresponding FL WinLab software (Perkin Elmer Ltd., UK) were used to indirectly assess bacterial adherence by measuring fluorescence emitted from monolayers and adherent FITC-labeled bacteria. The HT-29 monolayers with adherent bacteria were treated with 500 µl of trypsin:EDTA (0.25% w/v trypsin, 1 mM EDTA-4 Na) (Gibco) for 5 to 10 minutes at 37°C to remove the cells from the surface of the 24-well plate. The suspensions were transferred to 1.5 ml plastic disposable cuvettes for analysis. The luminescence spectrometer (Perkin Elmer) was set to an excitation wavelength of 494 nm and an emission wavelength of 519 nm that was specific for the quantitation of FITC.

3.7.8 Indirect Assessment of Bacterial Adherence Using Flow Cytometry

Lactobacillus spp. that exhibited adherence according to microscopic screening were selected to quantify adherence. The HT-29 monolayers and their adherent bacteria were subjected to treatment with 500 µl of trypsin:EDTA (0.25% w/v trypsin, 1 mM EDTA-4 Na) (Gibco) for 5 to 10 minutes at 37°C to remove the cells from the surface of the 24-well plate. The suspension was transferred to 5 ml Falcon polystyrene, round bottom tubes (Becton Dickinson Canada Inc., Ville Saint-Laurent, Quebec). Bacterial adherence was analyzed by flow cytometry (FACScan, Becton Dickinson). Preliminary analyses were conducted to determine optimal threshold values for side scatter and forward scatter. FACScan settings are shown in Table 3. Experimental values were calculated as a percent of the positive control.

3.7.9 Successive Washes to Remove Nonadherent Bacteria

Washes of the tissue monolayers were analyzed by flow cytometry (FACScan) to assess the optimum number of washes required to remove nonadherent bacteria from the assay. Side scatter and forward scatter thresholds were customized to be optimal for the measurement of bacteria. FACScan parameter values are shown in Table 3. Experimental values were calculated as a percent of the original incubation solution of the adherence assay.

3.8 Competition of *Lactobacillus* spp. with *Escherichia coli* strains

The *E. coli* strains selected for use in competition studies were *E. coli* ATCC 25922 and an *E. coli* strain isolated from a pig with diarrhea, designated *E. coli* PECI-80. *E. coli* ATCC 25922 was selected based on adherence visualized microscopically. *E. coli* PECI-80 was selected due to its role as a possible causative agent in pig diarrhea. Two methods for the assessment of competitive exclusion of the *E. coli* strains by the *Lactobacillus* strains were developed. Initially, the lactobacilli were labeled with FITC as described in Section 3.7.4 and the *E. coli* strains were labeled in a similar fashion with 0.01 mg/ml tetramethylrhodamine (TMR) (Molecular Probes, Inc). Two-color flow cytometry analysis was conducted to quantitate the adherence of both the lactobacilli and the *E. coli* strains. The alternate method for assessment of competition for adherence was performed using unlabeled lactobacilli and FITC-labeled *E. coli* strains. The adherence studies were performed as described in Sections 3.7.5 and 3.7.6, except for the following changes. The lactobacilli were allowed to adhere to the cell monolayer for 20 minutes at 37°C and nonadherent cells were removed by washing 2X with sterile PBS. The *E. coli*

Table 3. FACScan Parameter Settings for Flow Cytometric Analysis of Bacterial Adherence and Bacterial Washes

Sample Type	Parameter	Voltage	Amp Gain	Mode
Analysis of Adherence to HT-29 Cells	Forward Scatter	E-1	4.26	Linear
	Side Scatter	273	1.00	Linear
	FL-1 (FITC)	427	1.00	Log
Analysis of Bacterial Washes	Forward Scatter	E01	9.66	Log
	Side Scatter	463	1.00	Log
	FL-1 (FITC)	789	1.00	Log

cells were incubated with the monolayers for 20 minutes at 37°C and nonadherent cells were removed by washing 2X with sterile PBS. The above changes were made due to an observed tendency of the cell monolayer to lift from the surface of the plate after excessive manipulation. The monolayers and adherent bacteria were removed from the plates using trypsin:EDTA (Gibco) and analyzed using flow cytometry as described previously.

3.9 Experimental Design and Statistical Analysis

When studying the adherence of the lactobacilli to HT-29 monolayers, the experiment was set up as a randomized complete block in which each repetition was a block. This was done to account for variation in fluorescence values between repetitions. The data were later analyzed using SAS software. Orthogonal contrasts were performed between the group of experimental strains and the negative control and the group of experimental strains and the positive control.

4 Results

4.1 Characterization of Experimental Strains

4.1.1 Characterization of Experimental Strains

All porcine GIT isolates tested grew anaerobically on MRS at 37°C. They were gram positive, catalase negative and oxidase negative. Colony and cellular morphologies of the experimental strains are reported in Table 4.

4.1.2 Carbohydrate Fermentation Patterns by API CHL

The experimental strains were characterized for their fermentation profiles by API 50CH test strips. Most of the experimental strains were classified as either *Lactobacillus fermentum* or *Lactobacillus acidophilus*. Nine of twenty-three isolates are classified as *L. fermentum*, while seven of twenty-three isolates are *L. acidophilus* strains. The other isolates included one *Leuconostoc lactis*, one *Lactococcus lactis* subsp. *lactis* and several isolates that produced doubtful or unknown carbohydrate fermentation profiles. The API test classifications of the *L. fermentum* and *L. acidophilus* strains are shown in Table 4.

4.1.3 Plasmid Profiling of Experimental Strains

Isolation of plasmid DNA from *Lactobacillus* spp. was difficult and required extreme conditions to break the cells and release the DNA. Plasmid profiles were determined for *Lactobacillus acidophilus* and *Lactobacillus fermentum* strains. The objective of the plasmid profiling was to determine whether the same strains of *Lactobacillus* spp. were

Table 4. Physiological and Morphological Characteristic of Experimental Strains

Experimental Strain	Colony Morphology (MRS)	Gram Reaction	Idenitification According to API CH50
26LM	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
25IM	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
23JK	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
25CC	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
27JM	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
$27JM_B$	White, round, rough edged and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
24CK	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
22IM	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
25CK	White, round, smooth and entire	Gram positive; short, blunt rods in short chains or clumps	*Lactobacillus fermentum*
27IM	White, round, rough edged and entire	Gram positive; long thin rods in chains	*Lactobacillus fermentum*
22CM	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
$22CM_{B1}$	White, round, smooth and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
$22CM_{B2}$	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
21PM	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
24JC	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
23IM	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
26PC	White, round, rough edged and entire	Gram positive; medium length rods in chains	*Lactobacillus acidophilus*
22LC	White, round, rough edged and entire	Gram positive; long thin rods in chains	*Lactobacillus acidophilus*
21LC	White, round, rough edged and entire	Gram positive; long thin rods in chains	*Lactobacillus acidophilus*

found in multiple regions of the porcine GIT. Five of the *Lactobacillus fermentum* strains isolated from different sections of the porcine GIT produced the same seven-band plasmid profile (Figure. 1). The *L. fermentum* strain $27JM_B$ (lane 4) was later identified to be a rough colony variant of strain 27JM (lane 3), although it was initially differentiated on the basis of colony morphology. The experimental strains 27JM, 25CC, 25IM and 26LM (lane3, 5, 9 and 10, respectively) had identical plasmid profiles, indicating that these are most probably the same strain, which can colonize the jejunum, cecum, ileum and large intestine of the pig GIT. The *Lactobacillus acidophilus* strains were more difficult to study. Plasmid extraction methods were often unsuccessful. Only the *L. acidophilus* strains 22CM, $22CM_{B1}$ and $22CM_{B2}$ (lane 2,3 and 4, respectively) exhibited a common plasmid profile (Figure. 2). The *L. acidophilus* strains 22CM, $22CM_{B1}$ and $22CM_{B2}$ were initially differentiated solely on the basis of colony morphology, but were later identified as morphological variants of the same strain. Plasmid profiling results indicate that different strains of *Lactobacillus acidophilus* are found throughout the porcine GIT.

4.1.4 Bacteriocin Production by Experimental *L. acidophilus* and *L. fermentum* Strains

Bacteriocin production by selected strains of both *L. acidophilus* and *L. fermentum* was tested using several different indicator organisms, including strains of *Listeria* and *Lactobacillus*. Direct and deferred inhibition, as well as spot-on-lawn experiments produced variable results. None of the experimental strains consistently produced defined zones of inhibition. Zones that were visible were small, and characteristic of an

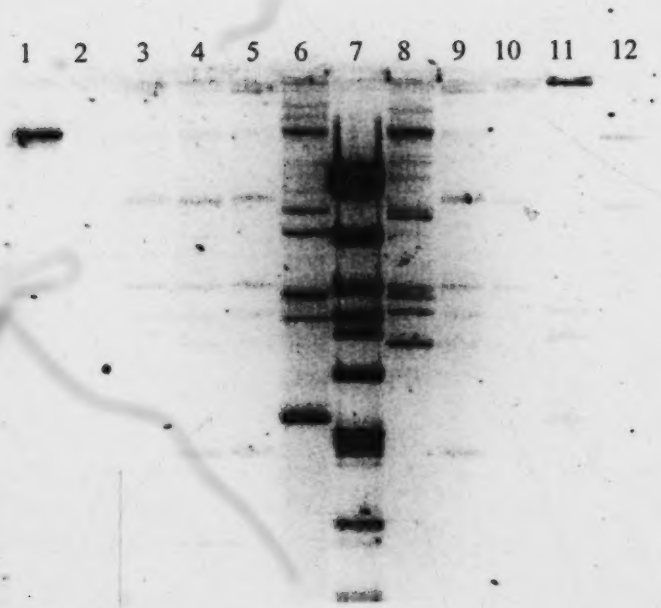


Figure 1. Plasmid profiling of experimental *L. fermentum* strains

[lane 1=extraction control *Carnobacterium piscicola* UAL 8, lane 2=27IM, lane 3=27JM, lane 4=27JM$_B$, lane 5=25CC, lane 6=25CC$_B$, lane 7=cla λ DNA ladder, lane 8=25CK, lane 9=25IM, lane 10=26LM, lane 11=22IM, lane 12=24CK]

Figure 2. Plasmid profiling of experimental L. acidophilus strains
[lane 1=extraction control Carnobacterium piscicola UAL 8, lane 2=22CM, lane 3=22CMB1, lane 4=22CMB2, lane 5=cla λ DNA ladder, lane 6=22LM, lane 7=21LM, lane 8=24JK, lane 9=extraction control Carnobacterium divergens UAL 9]

inhibitory effect due to acid production. Protease activity on the inhibitory effect was not tested due to the size and inconsistency of zone production.

4.2 Acid Tolerance of Selected *L. acidophilus* and *L. fermentum* strains

4.2.1 Plate Counts

Three *L. fermentum* strains and two *L. acidophilus* strains were screened for acid tolerance. Preliminary studies using plate counts to assess bacterial survival at different pH values demonstrated that most strains tested survived control conditions as well as incubation in pH 3.5 MRS at 37°C for 24 hours and pH 2.5 MRS at 37°C for three hours. However, none of the strains survived at counts above $1x10^1$ cfu/ml (the detection limit) in pH 1.5 MRS (Table 5).

4.2.2 Absorbance Measurements

All three of the *L. fermentum* strains and both of the *L. acidophilus* strains were selected on the basis of the preliminary results in Section 4.2.1 for further study of their acid tolerance. These strains were incubated at 37°C in MRS media adjusted to pH 1.5, 2.5 and 3.5. Growth was assessed using a microplate reader. The *L. fermentum* strains had similar growth curves, as did the *L. acidophilus* strains. However, the *L. fermentum* strains consistently grew more rapidly than the *L. acidophilus* strains under control conditions. Representative growth curves are shown in Figure 3. None of the bacterial strains tested was able to grow at pH 3.5, pH 2.5 or pH 1.5.

Table 5. Plate Counts (log number cfu/ml) of Selected Lactobacilli After Exposure to Low pH*

		Time of Incubation (hrs)			
pH	**Strain**	**0**	**1.5**	**3**	**24**
pH 6.8 (control)	27JM	7.1	7.2	7.5	9.5
	26LM	7.1	7.2	7.4	9.4
	25IM	7.0	7.2	7.4	9.3
	23IM	6.4	6.2	6.5	8.7
	21PM	6.7	6.5	6.8	9.0
pH 3.5	27JM	7.3	7.2	7.1	5.8
	26LM	7.1	7.3	7.2	6.1
	25IM	7.1	7.1	7.1	6.5
	23IM	7.1	6.6	6.3	6.0
	21PM	6.5	7.0	6.9	5.9
pH 2.5	27JM	7.1	7.3	7.1	<1
	26LM	7.0	7.2	6.9	<1
	25IM	7.1	7.0	7.0	<1
	23IM	6.6	5.7	< 6	<1
	21PM	6.6	5.2	< 6	<1
pH 1.5	27JM	7.1	<1	<1	<1
	26LM	7.2	<1	<1	<1
	25IM	7.3	<1	<1	<1
	23IM	6.6	<1	<1	<1
	21PM	6.6	1.0	<1	<1

* data represent average values from duplicates within four replicates

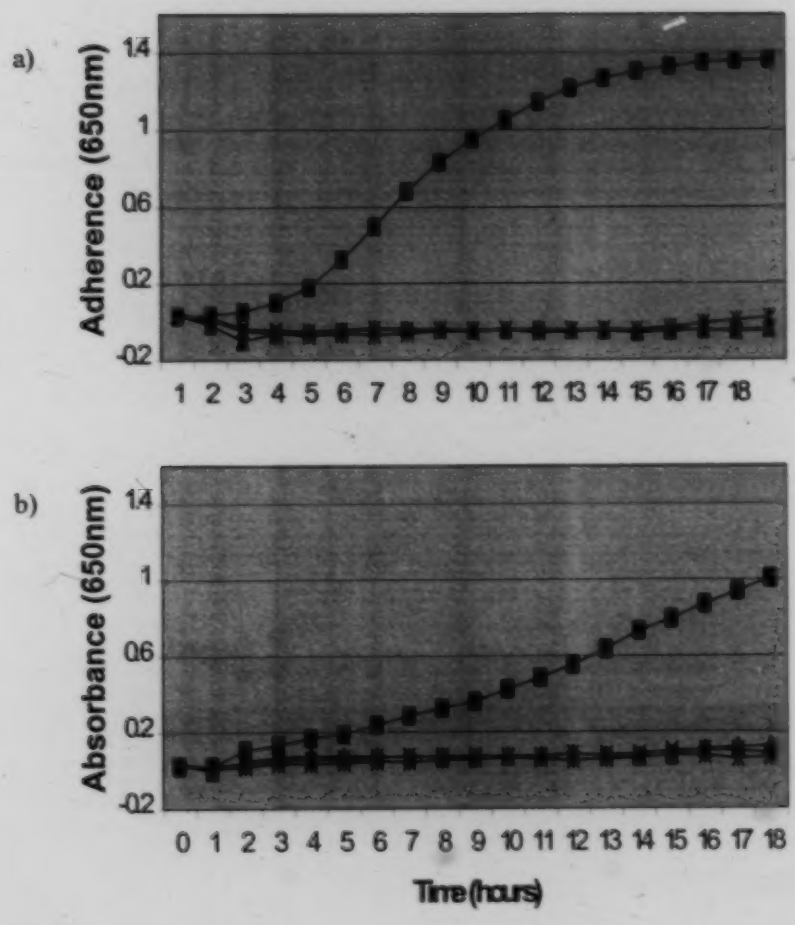


Figure 3. Acid Tolerance of a) *L. fermentum* 27JM and b) *L. acidophilus* 23IM [control MRS broth pH 6.8 (■), pH 1.5 (▲), pH 2.5 (X), pH 3.5 (✱)]

4.3 Bile Tolerance of Selected *L. acidophilus* and *L. fermentum* strains

4.3.1 Preliminary Bile Tolerance

The *L. fermentum* and *L. acidophilus* strains tested for acid tolerance were screened for bile tolerance in MRS broth with various concentrations (0, 0.15, 0.3, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10% w/v) of Oxgall (bMRS). Turbidity of the broth was observed visually at 24, 48 and 72 hours to assess bacterial growth. All strains exhibited good growth at all bile concentrations. Slower growth was observed at higher concentrations of bile.

4.3.2 Bile Tolerance

Due to good growth characteristics in bMRS, all five of the *Lactobacillus* strains were further evaluated for bile tolerance. Growth in 0%, 0.3% and 1.5% bMRS was evaluated using a microplate reader. As previously discussed in Section 4.2, stains of each species had similar growth curves. The *L. fermentum* strains grew faster than the *L. acidophilus* strains. Representative curves shown in Figure 4 demonstrate that both *L. fermentum* and *L. acidophilus* strains grow well at all bile concentrations. The *L. fermentum* strains, in particular, grew equally well under all experimental conditions.

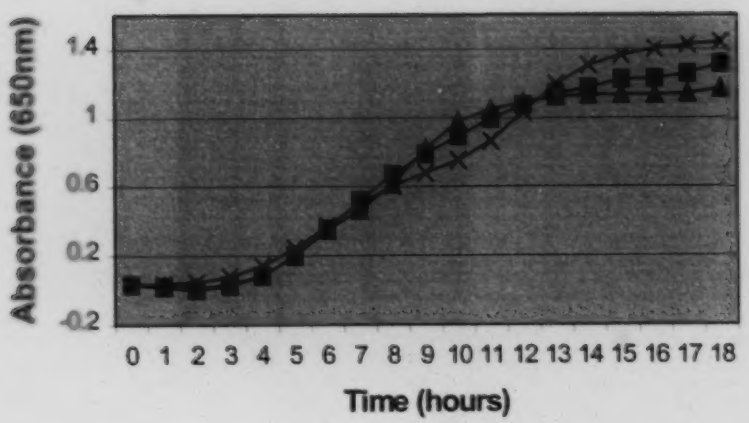
4.4 Sequential Acid and Bile Tolerance

The five *Lactobacillus* strains that exhibited good acid and bile tolerance were screened for tolerance to sequential acid and bile exposure, simulating conditions in the GIT. All five strains grew under all experimental conditions (Figure 5). It is interesting to note that all five of the strains grew well in pH 3.5 MRS which was neutralized after 1.5

hours. These data contradict the results reported in Section 4.2.2, where the strains were unable to grow when exposed to acidic conditions for up to 18 hours. However, these data, taken with those presented in Section 4.2.1, demonstrate that the selected strains can survive acidic conditions for a short time, but cannot survive and grow when exposed to acidic conditions for a longer time.

The *L. fermentum* strains exhibit a faster rate of growth than the *L. acidophilus* strains under all conditions, although the maximum population reached by the stationary phase was similar. In general, therefore, the *L. fermentum* strains grew faster than the *L. acidophilus* strains.

a)



b)

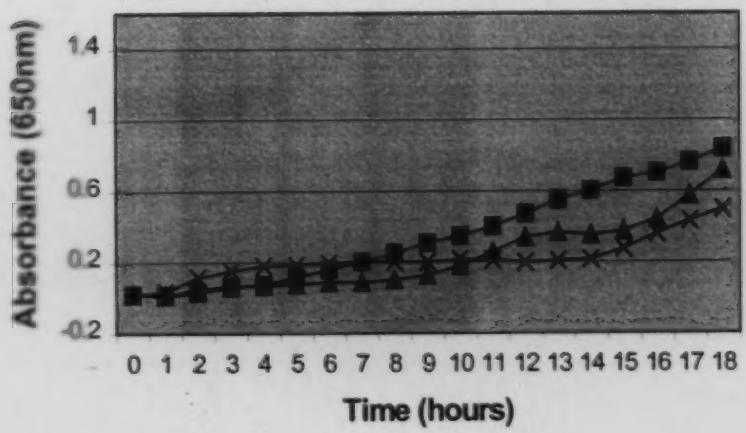
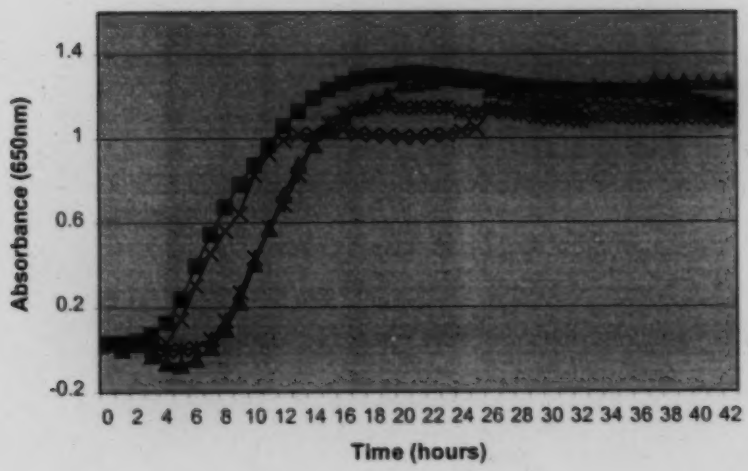


Figure 4. Bile Tolerance of a) *L. fermentum* 27JM and b) *L. acidophilus* 23IM [control MRS broth 0% Oxgall (■), 0.3% Oxgall (▲), 1.5% Oxgall (X)]

a)



b)

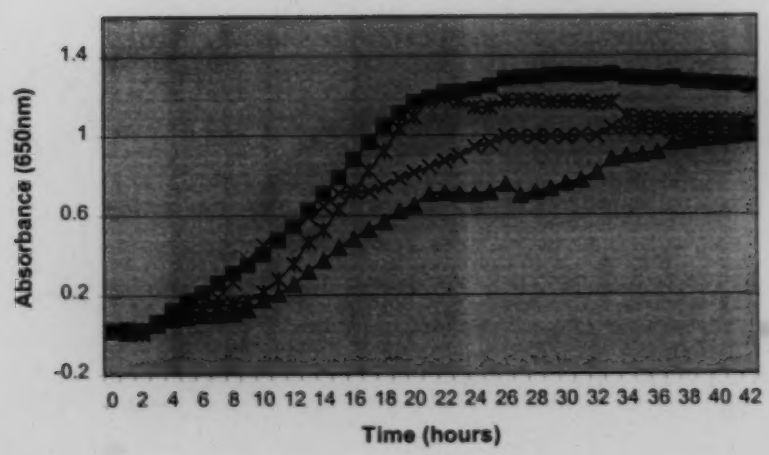


Figure 5. Sequential Acid and Bile Tolerance of a) *L. fermentum* 27JM and b) *L. acidophilus* 23IM [control MRS broth pH 6.8 + 0% Oxgall (■), pH 3.5 + 0% Oxgall (▲), pH 6.8 + 0.2% Oxgall (X), pH 3.5 + 0.2% Oxgall (✱)]

4.5 Bacterial Adherence to Human HT-29 Cells

4.5.1 Growth of the Cell Line

The HT-29 cell line formed a confluent monolayer in tissue culture flasks and in the 24-well plates in approximately two to three days. The cells were easily detached from the polystyrene surface and from each other using trypsin digestion. Reattachment to the polystyrene surface was exhibited within minutes of passaging.

4.5.2 Preliminary Study of Bacterial Adherence

The five *Lactobacillus* strains previously selected also were screened for adherence to HT-29 monolayers. Adherence was examined visually using a fluorescence microscope and assessed qualitatively. *L. fermentum* strains 27JM, 26LM and 25IM, and *L. acidophilus* strain 23IM adhered to the monolayer, while the *L. acidophilus* strain 21PM exhibited relatively poor adherence. As described in Section 4.1.3, 26LM, 25IM and 27JM were found to be the same strain according to plasmid profiling. Both 27JM and 25IM were chosen to represent the dominant strain of *L. fermentum*, while 23IM was chosen as an adherent strain of *L. acidophilus*. These selected strains were used for further adherence experiments.

Several other strains of lactobacilli and *E. coli* were assessed visually for adherence to HT-29 cells. *L. acidophilus* BG2 FO4 showed strong adherence to the cell line, while *L. plantarum* 4008 showed poor adherence. Therefore, these strains were chosen as positive

and negative controls, respectively. *E coli* strains MH1 and DH5α exhibited very little adherence, while ATCC strains 11229 and 25922 exhibited some adherence to undifferentiated HT-29 cells. Based on observed numbers of bacteria adhering per cell, *E. coli* ATCC 25922 demonstrated greater adherence than ATCC 11229 and was, therefore, chosen for further study as discussed in Section 4.5.6.

4.5.3 Method Development for Indirect Assessment of Adherence

As described in Section 4.5.2, the use of fluorescence microscopy for direct assessment of adherence to HT-29 monolayers was useful for the identification of controls and adherent experimental strains. However, counting the number of bacteria per HT-29 cell proved to be extremely labor intensive and the probability of counting error was deemed to be high. Also, the FITC fluorescence faded rapidly under microscopic analysis making counting almost impossible. Using the luminescence spectrometer to measure adherence indirectly was initially useful. However, fluorescence values obtained varied greatly between duplicates within repetitions and individual repetitions. Clumping of the HT-29 cells often disturbed the uniform turbidity of the cell suspension, therefore, distorting fluorescence values. The cells had a tendency to fall to the bottom of the cuvette, making time of evaluation a factor. Inconsistencies necessitated the development of another method. As described in more detail in Section 4.5.5, the use of flow cytometry to assess indirectly bacterial adherence to HT-29 monolayers provided the most consistent and reliable results.

4.5.4 Successive Washes to Remove Nonadherent Bacteria

Tissue culture monolayers with adherent bacteria were washed with 1 ml of sterile PBS to remove the nonadherent bacteria. Preliminary adherence studies included the analyses of several washes to determine the number of washes that would adequately remove the majority of nonadhering bacteria. These results are illustrated in Figure 6. Flow cytometry measurements indicate that three washes with 1 ml sterile PBS was adequate to remove most of the unbound FITC from the bacterial pellet. Figure 6 demonstrates that the fourth wash contains less than 4% of the fluorescence of the initial adherence suspension. The negative control was a wash of a monolayer without the addition of the bacterial suspension. Its fluorescence averaged less than 4% of the initial incubation suspension. These data indicate that the fourth wash may simply contain tissue culture cells and debris that are emitting background fluorescence. Washing the tissue culture monolayer more than three times caused the monolayer to be removed from the surface of the 24-well plate. Thus, three washes were consistently used to remove nonadherent bacteria from the tissue culture monolayer.

4.5.5 Adherence of Lactobacilli to HT-29 Cells

As described in Section 4.5.3, flow cytometric analysis of the cells and their adherent bacteria proved to be the most useful. Three *Lactobacillus* strains, *L. fermentum* 27JM, *L. fermentum* 25IM and *L. acidophilus* 23IM, were chosen for adherence studies due to the exhibition of good adherence properties when examined microscopically. When assessed using flow cytometry, the selected strains demonstrated high adherence,

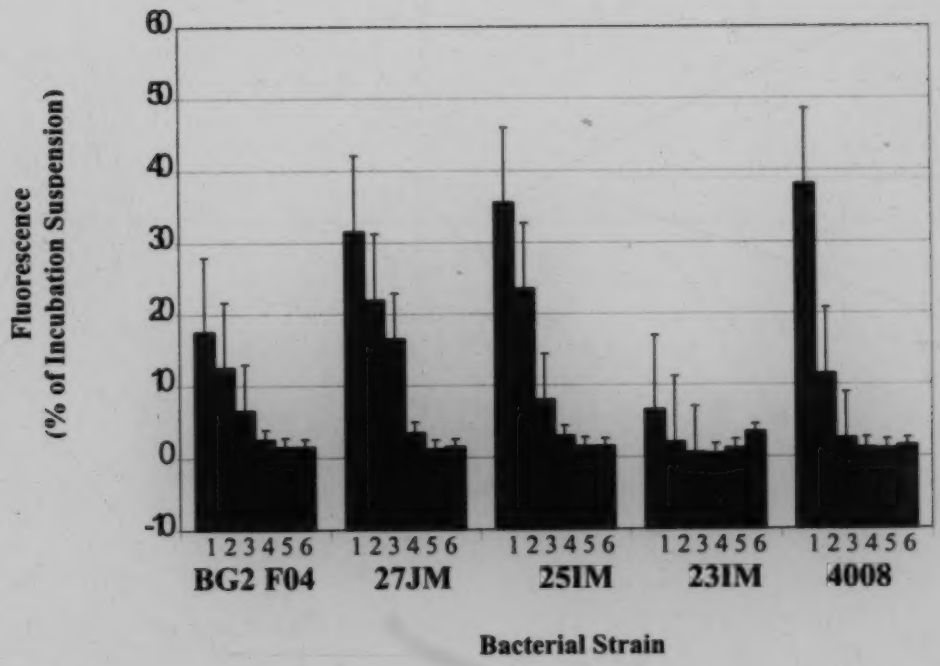


Figure 6. Flow cytometric analysis of successive washes of HT-29 monolayers and adherent bacteria to remove nonadherent bacteria. The data are represented as a percentage of the maximum fluorescence of the experimental bacterial incubation suspension which was set at 100%. [1 = wash 1, 2 = wash 2, 3 = wash 3, 4 = wash 4, 5 = wash 5, 6 = blank (tissue culture incubated without bacterial suspension)]

measured by the intensity of fluorescence emitted from the washed tissue culture monolayers with adherent FITC-labeled bacteria. Figure 7 illustrates the adherence of the experimental strains, as a percentage of the control value. *L. fermentum* strains 27JM and 25IM and *L. acidophilus* strain 23IM all exhibited good adherence to the HT-29 cells, similar to that of the positive control and significantly greater than the negative control and the blank background fluorescence. Orthogonal contrasts indicate that the adherence of the experimental strains is significantly different from the adherence of the negative control ($p<0.01$). However, the adherence of the experimental strains is not significantly different than the adherence of the positive control ($p=0.16$).

4.5.6 Competitive Exclusion of *E. coli* by Lactobacilli

As previously noted, *E. coli* ATCC 25922 showed adherence to the HT-29 tissue culture when examined microscopically. Another strain of *E. coli* (PECI-80) was isolated from a piglet with diarrhea and was included in the exclusion study. Although preliminary microscopic results were promising for strain ATCC 25922, both *E. coli* strains exhibited low fluorescence values during flow cytometry compared with data for adherence of the lactobacilli (Figure 8). Numbers of *E. coli* were increased to $1x10^{11}$ to $1x10^{12}$ cfu/ml to increase their adherence during the competitive exclusion study.

The competition between lactobacilli and *E. coli* was examined using two fluorescence methods. The first employed the incubation of the monolayers with FITC-labeled lactobacilli and subsequent incubation with TMR-labeled *E. coli*. The two-color analysis of these data was complex. Several controls were required and the data was difficult to

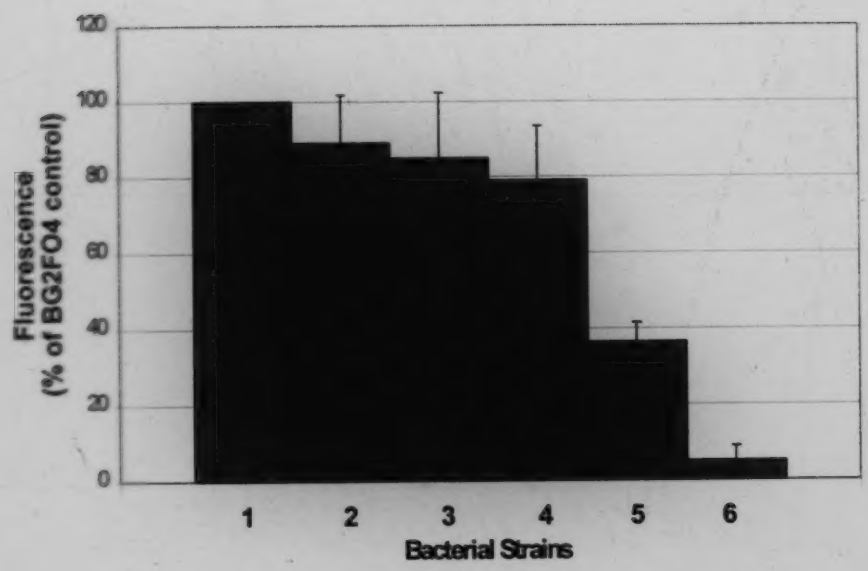


Figure 7. Flow cytometric analysis of bacterial adherence to human HT-29 tissue culture monolayers
[1 = positive control *L. acidophilus* BG2 FO4, 2 = *L. fermentum* 27JM, 3 = *L. fermentum* 25IM, 4 = *L. acidophilus* 23IM, 5 = *L. plantarum* 4008, 6 = HT-29 cell culture blank]

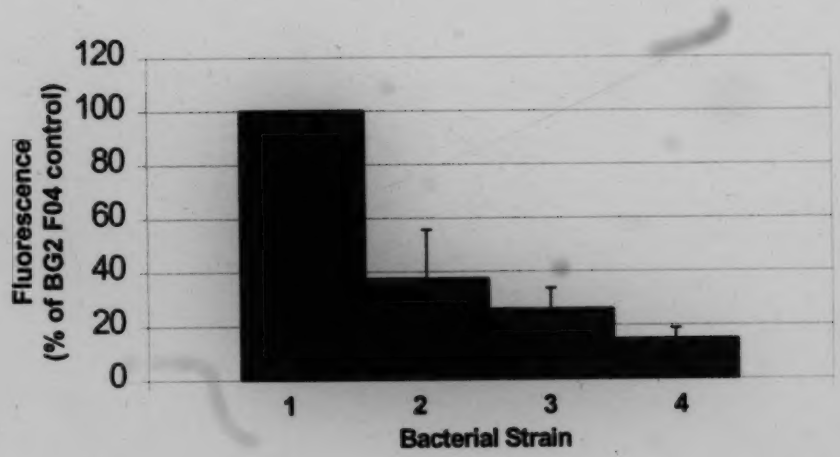


Figure 8. Comparison of the adherence of control lactobacilli and experimental *E. coli* strains, as assessed by flow cytometry, as a percent of the positive control *L. acidophilus* BG2 F04
[1 = *L. acidophilus* BG2 FO4, 2 = *L. plantarum* 4008, 3 = *E. coli* ATCC 25922, 4 = *E. coli* PECI-80]

interpret. When TMR-labeled *E. coli* were incubated with the HT-29 monolayers and adherent FITC-labeled lactobacilli, the FITC fluorescence values were greater than when the monolayers were incubated only with FITC-labeled lactobacilli. That is, FITC intensity was increased even without the addition of FITC. Due to concern about the overlap in detection of the two stains, flow cytometric analysis requiring only one of the two stains was preferred. Therefore, the *E. coli* were labeled with FITC and the lactobacilli remained unlabeled. The fluorescence values of the HT-29 cells, incubated with both lactobacilli and *E. coli*, could then be compared with those incubated with *E. coli* alone. It was decided that this would be useful in calculating differences in adherence.

When the HT-29 cell monolayers were incubated with control and experimental strains of lactobacilli prior to incubation with *E. coli* strains, the fluorescence values were 3 to 4 times higher than those when the monolayers were incubated with lactobacilli alone. However, the fluorescence values for the *E. coli* alone, remained low. It is also interesting to note that Lactobacillus plantarum 4008, which demonstrates poor adherence to the HT-29 monolayers, also increased the apparent adherence of the *E. coli* strains. These results (Figure 9) were unexpected, but they were consistent in four repetitions of the experiment using two strains of *E. coli* and five strains of *Lactobacillus* spp. in the preincubation.

A histogram of cell counts, or events, versus fluoresence is shown in Figure 10. The histogram is representative of most histograms from all the adherence experiments. As

illustrated, most samples of HT-29 cells and their adherent bacteria produced sharp peaks of average fluorescence, indicating a homogeneous cell population. However, on rare occasions, during the competition studies a histogram with a dual peak was produced. This may indicate the preferential adherence of the bacterial cells to a particular population of tissue culture cells. However, it may simply indicate clumping of the tissue culture cells and adherent bacteria producing artificially high fluorescence values.

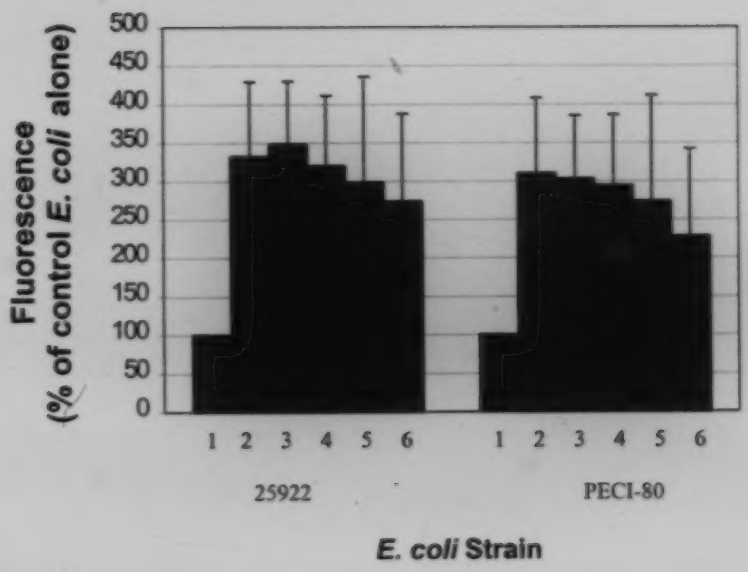


Figure 9. Flow cytometric analysis of *E. coli* ATCC 25922 and PECI-80 to HT-29 monolayers pre-incubated with strains of lactobacilli, as a percent of control *E. coli* adherence
[1 = *E. coli* strain alone, 2 = pre-incubation with *L. acidophilus* BG2 FO4, 3 = pre-incubation with *L. fermentum* 27JM,
4 = pre-incubation with *L. fermentum* 25IM, 5 = pre-incubation with *L. acidophilus* 23IM, 6 = pre-incubation with *L. plantarum* 4008]

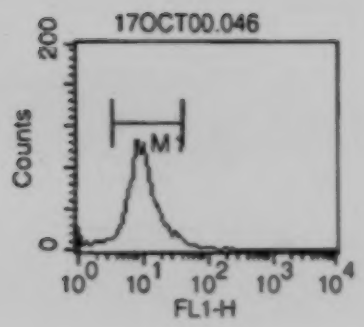


Figure 10. Flow cytometric histogram of cells counts versus FITC fluorescence (FL1-H) M1 marks the population used to calculate the average FITC fluorescence of the HT-29 cells and their adherent bacteria. The nice, sharp peak indicates the homogeneity of the fluorescence of the cell population

5. Discussion and Conclusions

Normal gut microflora have demonstrated abilities to prevent GIT infection in animals and humans. Nurmi and Rantala (1973) prevented *Salmonella* infection by dosing chicks with the intestinal contents of healthy adult chickens. This phenomenon of colonization resistance is important in the development of useful probiotics for humans and animals. It is important that the probiotic strains are able to tolerate the extreme acid and bile conditions of the gut. Also, it is well documented that colonization of the gut by a bacterial strain is essential for probiotic activity. Several studies have established that selected *Lactobacillus* spp. are resistant to acid and bile. Although the adherence of *Lactobacillus* spp. to intestinal epithelial cells *in vitro* and *in vivo* has been demonstrated, until recently, relatively little was known about the mechanisms of adherence of the lactobacilli. The objective of this study was to develop a set of rapid, simple *in vitro* experiments to investigate several of the probiotic properties of *Lactobacillus* strains. These methods were then implemented in the characterization of *Lactobacillus* strains isolated from the GIT of a pig as potential probiotics. Future considerations include the use of a probiotic strain to prevent *E. coli* scours in piglets.

Initial purification of pig GIT isolates was difficult because of two colony variations within several of the cultures. Rough and smooth colony variants were observed for several isolates. The rough variants formed colonies on agar media that were rough, flat and irregular, while the smooth variants formed colonies that were smooth, regular and convex. Colony variants have been observed for several *Lactobacillus* spp., including *L.*

fermentum (Henriksson et al., 1991) and *L. acidophilus* (Klaenhammer and Kleeman, 1981).

L. fermentum and *L. acidophilus* have been shown to be the most dominant lactobacilli in adult, weanling and suckling pigs (Fuller et al., 1978; Pedersen and Tannock, 1989; Naito et al., 1995). The current study supports these findings in that nine of twenty-three GIT isolates were characterized as *L. fermentum* and a further seven isolates were characterized as *L. acidophilus*. Five sections of the pig GIT were sampled: pars esophagea, ileum, jejunum, cecum and large intestine. All of these surfaces consist of squamous epithelial cells. *L. fermentum* strains were found in four of the five areas sampled, including the ileum, jejunum, cecum and the large intestine. However, strains of *L. acidophilus* were found in all five of the sections sampled. Although the *L. acidophilus* strains are more widespread, plasmid-profiling results indicated the probability that a single strain of *L. fermentum* colonized the ileum, jejunum, cecum and large intestine. In contrast, the five sections of the pig GIT appeared to be colonized by different strains of *L. acidophilus*.

Based on plasmid profiles and the pathogenic importance of adherence of *E. coli* strains to pig small intestinal enterocytes (Blomberg et al., 1993; Casey et al., 1992; Ouwehand and Conway, 1996), *L. fermentum* 27JM and 25IM, and *L. acidophilus* 23IM were selected for further investigation. Chou and Weimer (1999) demonstrated that all of the *L. acidophilus* strains tested tolerated pH 3.5 for 90 minutes. Also several strains of *L. salivarius* isolated from the GIT of chicks demonstrated tolerance to pH 3.0 for three

hours (Garriga et al., 1998). This study supports these data in that all of the *L. acidophilus* and *L. fermentum* strains tested produced viable plate counts, comparable to those of the control (pH 6.8), after exposure to pH 3.5 for 1.5 and 3 hours (Table 5). As the pH was reduced the counts decreased to below the detection limit of 10^1 cfu/ml, for example, when the cells were incubated at pH 1.5 for 1.5 hours or longer. However, when growth was assessed using a microplate reader, none of the strains was able to grow under any conditions other than the control conditions (Figure 3). Chou and Weimer (1999) reported that none of the strains that tolerated pH 3.5 for 90 minutes could form colonies on acidified MRS after 96 hours. These data suggest that certain strains may survive short-term acid exposure, but not long-term exposure.

Several studies have proven that the lactobacilli are often resistant to bile (Chou and Weimer, 1999; Garriga et al., 1998). The concentration of bile in the gut of an animal at any one time varies depending on time and composition of last food intake, as well as the animal's health and individual variation. Therefore, it is difficult to define a standard concentration to use to indicate bile tolerance. Bile tolerance was initially indicated by the ability of the organism to grow in 0.15% w/v bile salts; however, resistant strains are now often tested using 0.3% w/v bile salts. Studies have differed in the composition of the experimental bile used, from commercial products (Oxgall) to a combination of individual bile salts. This study supported the data that several *Lactobacillus* strains are resistant to bile, using 0.3% w/v Oxgall and even a more extreme 1.5% w/v Oxgall (Figure 4).

Staining the adherent bacterial cells using Gram staining or Giemsa staining methods, for example, and directly counting the number of adherent bacteria per epithelial cell have often been used to quantify adherence. However, these methods are time consuming, labor intensive and prone to counting error. Therefore, a more reliable and efficient method is desirable. In this study, the use of a luminescence spectrometer to quantify fluorescence was examined. The results were inconsistent and showed high variation between duplicates. Also, the concept of the 'black box', where a sample is inserted and a single numerical value is produced was not desirable for the quantification of a process as complex as adherence. That is, more information about the cell culture monolayer and adherent bacteria would be an asset. Flow cytometry has the advantage of extensive data output, including fluorescence emission and information about the tissue culture cell population. The wealth of information to be gained, as well as the efficiency of flow cytometry made this method invaluable in the indirect measurement of bacterial adherence to tissue culture cells.

Logan et al. (1998) developed a flow cytometric assay to quantitate the adherence of *Helicobacter pylori* to gastric epithelial cells. The researchers labeled their bacteria with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE), a fluorescein derivative. Data obtained by flow cytometric analysis was used to determine the proportion of gastric cells with adherent *H. pylori* and the mean number of adherent bacteria per cell. Clarke and Morton (2000) developed an *in vitro* fluorometric assay, using FITC labeled bacteria, to study the adherence of *Pasteurella haemolytica* to bovine epithelial cells. Modified methods were used in this study in an attempt to quantify the adherence of

Lactobacillus spp. and *E. coli* to monolayers of HT-29 epithelial cell culture. Results indicate that flow cytometric analysis is a useful method for the indirect assessment of *Lactobacillus* spp. adherence to undifferentiated HT-29 cell monolayers (Figure 7). Further investigation of the adherence of *E. coli* strains to undifferentiated cell lines, as well as their interactions with other bacterial strains, including lactobacilli, is required.

Several studies have demonstrated the adherence of lactobacilli to tissue culture cell lines *in vitro* (Chauvière et al., 1992; Greene and Klaenhammer, 1994; Sarem et al., 1996; Granato et al., 1999). For instance, Caco-2 cells have commonly been used to assess bacterial adherence. Caco-2 and HT-29 cell lines originated from human colon carcinomas and form differentiated, polarized monolayers with tight junctions and microvilli when incubated for an average of fifteen days. Thus, they are thought to be representative of the squamous epithelial cell layer of the GIT (Chauvière et al., 1992). However, in this study a more rapid method of assessing adherence was desired, therefore the HT-29 cells were incubated for an average of 2 days. Only long enough to form a confluent monolayer of undifferentiated cells. The lactobacilli tested in this study adhered to the undifferentiated HT-29 monolayer indicating that an undifferentiated cell line is also useful for *in vitro* adherence studies.

The interest in the use of probiotic supplements to inhibit colonization of the GIT by intestinal pathogens is increasing. Preventing scours resulting from *E. coli* infection in pigs is an important concern in the swine industry. *E. coli* is one of the main causative agents of diarrhea in the swine industry (Fitzgerald et al., 1988; Johnson et al., 1992).

The prevalence of *E. coli* infection causes serious health concerns for both animals and humans.

E. coli adherence has been well studied and is shown to use several specific adhesins, as well as a combination of nonspecific mechanisms to adhere to intestinal epithelial cells *in vivo* and *in vitro*. The human Caco-2 cell line has effectively been used to assess the adherence of *E. coli* strains *in vitro*. As discussed earlier, the Caco-2 and the HT-29 cell lines are similar in that they can both form differentiated monolayers after prolonged incubation. In this study, the *E. coli* strain ATCC 25922 demonstrated good adherence to the undifferentiated HT-29 monolayer when observed using fluorescence microscopy. However, further investigation using flow cytometry techniques indicated that neither *E. coli* ATCC 25922 nor *E. coli* PECI-80 adhered well to the undifferentiated HT-29 monolayer (Figure 8). It is well understood that *E. coli* are able to bind to differentiated cell lines due to the expression of specific receptors on the tissue culture. However, this study used undifferentiated cell monolayers. Under the assumption that the *E. coli* cells could bind to these monolayers using nonspecific mechanisms, high numbers of *E. coli* cells were added to HT-29 monolayers in the adherence assay in an attempt to increase adherence. The lower binding of the *E. coli* strains to the undifferentiated cell line is consistent with information in the current literature, in that *E. coli* strains use several specific adhesins to bind to receptors that would be better expressed in the differentiated system.

Conflicting evidence currently exists with respect to the inhibition of *E. coli* adherence by *Lactobacillus* spp. Spencer and Chesson (1994) demonstrated that the adherence of *Lactobacillus* spp. prior to incubation with *E. coli* strains was ineffective in preventing the adherence of the *E. coli* to isolated porcine enterocytes. However, Chauvière et al. (1992) and Coconnier et al. (1993) inhibited *E. coli* adherence to Caco-2 cells in a concentration-dependent manner using living and heat-killed cells of *L. acidophilus* strain LB. Other studies have demonstrated that a compound released by *L. fermentum* 104 into the spent culture supernatant inhibited the adherence of *E. coli* strains to piglet ileal mucus (Blomberg et al., 1993; Ouwehand and Conway, 1996). The competitive exclusion appears to be associated with the specific binding of *E. coli* expressing the K88 adhesin to specific receptors in the ileal mucus. The present study not only demonstrated the inability of the *Lactobacillus* strains to prevent *E. coli* adherence, but also indicated that the lactobacilli may enhance *E. coli* adherence to HT-29 monolayers. Spencer and Chesson (1994) observed a coaggregation phenomenon between the lactobacilli, especially *L. fermentum*, and enterotoxigenic *E. coli*. Interpretation of the present study would also suggest that coaggregation is occurring between the test strains of *Lactobacillus* and *E. coli* strains, thereby increasing the adherence of the *E. coli* strains. The reason that the low adherence control *L. plantarum* 4008 also increases the adherence of *E. coli* is unclear. Further study of this phenomenon is required. It is important to note that *in vivo* conditions are different from *in vitro* adherence assays in that there is a constant flushing of the system with digesta and sloughing of epithelial cells. In this way the coaggregation of the *E. coli* to the lactobacilli may provide an effective trapping mechanism, ridding the host of *E. coli* cells before they are able to

adhere and cause infection. In fact, Reid et al. (1988) indicated that the coaggregation of probiotic lactobacilli with uropathogenic *E. coli* provided an important host defense mechanism against infection. *L. fermentum* strain A-60 was shown to coaggregate with P-fimbriated *E. coli*, as well as type 1 fimbriated *E. coli*, including *E. coli* ATCC 25922.

Further support that experimental lactobacilli used in this study may be useful in the prevention of *in vivo E. coli* infection in pigs exists (personal communication, ME Stiles). *L. fermentum* strain 25IM, used in this study, was genetically modified to produce Colicin V (Col V), a bacteriocin effective against *E. coli*. The Col V producer strain and the wild type strain were fed to experimental pigs in an attempt to prevent subsequent infection by ingested *E. coli*. The Col V producer strain prevented diarrhea in all of the five pigs subsequently challenged with *E. coli*. Col V nonproducers prevented diarrhea in two of five pigs, while all five of the untreated pigs developed diarrhea. Thus, the *L. fermentum* strain 25IM provides a preventative protection against *E. coli* infection *in vivo*.

Modified methods to determine acid, bile and sequential acid and bile tolerance of *Lactobacillus* strains isolated from pig GIT were developed. A flow cytometric method using FITC-labeled bacteria was developed for the indirect assessment of the adherence of these *Lactobacillus* strains to undifferentiated HT-29 monolayers. These methods proved to be rapid, simple and efficient. Also, the *Lactobacillus* strain isolated from the pig GIT proved to be resistant to short-term acid treatment, bile treatment and sequential acid and bile treatment according to the methods developed. Selected strains were

adherent to undifferentiated HT-29 cell monolayers. Thus, the methods developed may be useful for assessing the probiotic potential of other *Lactobacillus* strains.

This study has provided further evidence of the probiotic potential of both *L. fermentum* and *L. acidophilus* strains. Also, a series of rapid and simple experiments has been compiled, modified and developed, to allow for easy identification of potentially probiotic organisms. Further study of the adherence mechanisms of the lactobacilli and the interactions between the lactobacilli and *E. coli* would be of great interest.

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